Chapter 1

ANALYTICAL SUPERCRITICAL FLUID CHROMATOGRAPHY AND EXTRACTION

Jerry W. King, Herbert H. Hill, Jr., and Milton L. Lee

Supplement and Cumulative Index, Edited by Bryant W. Rossiter and Roger C. Baetzold. Physical Methods of Chemistry Series, 2nd ed., Vol. X.
ISBN 0-471-57086-9

1 INTRODUCTION

1.1 Definitions

Chromatography and extraction are two closely related analytical processes used extensively for chemical separation and isolation. Both rely on the distribution of an analyte between two phases, a separating phase and stationary phase. In extraction, the separating phase is commonly referred to as the extracting phase and the sample as the stationary phase. In chromatography, the separating phase is called the mobile phase and the stationary phase is an immobilized liquid or solid phase over which the mobile phase passes. Quantitatively the distribution of an analyte between two phases can be expressed as

$$K = \frac{C_s}{C_m} \tag{1}$$

where K is called the partition coefficient, C_m represents the concentration of the analyte in the mobile (or extracting) phase, and C_s represents the concentration of the analyte in the stationary phase. In extraction, this distribution is used to separate the analyte from the sample. In chromatography, compounds with different K values can be isolated from each other through repetitive distributions between a separating (mobile) phase passing over a stationary phase.

The most common separating phases have been liquids and gases. Liquid extraction and liquid chromatography (LC) are methods in which the separating phase is liquid, while in distillation and gas chromatography (GC) the separating phase is a gas. When supercritical fluids are used as the separating phase rather than gases or liquids, the separation processes are called supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC).

The definition of a supercritical fluid is best described by using a typical pressure–temperature phase diagram as shown in Figure 1.1. Above the critical pressure of a substance, a phase transition to a gaseous state is no longer observed as the liquid form of the substance is heated. Similarly, above the critical temperature of a substance, a phase transition to a liquid state is no longer observed as the gaseous form of the substance is pressurized. In the region above the critical temperature and pressure, a substance can no longer be classified as either a gas or a liquid since it has properties of both. In this region above the critical temperature and pressure, a substance is said to be a supercritical fluid. From a practical point of view, supercritical fluids can be thought of as gases that have been compressed to densities at which they can exhibit liquid-like interactions.

1.2 Characteristics

It is both the liquid-like and gas-like characteristics of supercritical fluids that make them unique for chemical separation. In particular, supercritical fluid

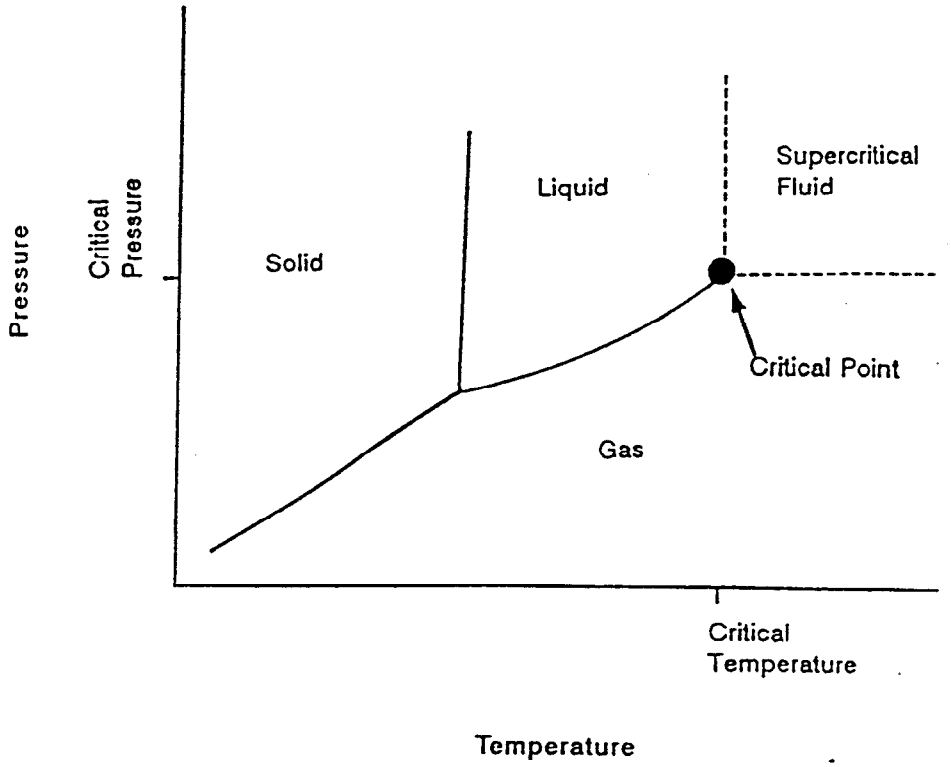

Figure 1.1 Pressure–temperature phase diagram demonstrating the supercritical fluid region and its relation to liquid- and gas-phase regions.

densities, diffusivities, and viscosities fall into ranges between those of liquids and gases. Under practical analytical operating conditions, pressures from 50–500 atm and temperatures from ambient to 300°C, densities of supercritical fluids range from one to eight-tenths of their liquid densities. Diffusivities of analytes in supercritical fluids throughout this operating range vary between 10^{-3} and 10^{-4} cm^2/s compared to values of less than 10^{-5} cm^2/s for liquids. Viscosities of supercritical fluids are typically 10–100 times less than those of liquids.

On the other hand, viscosities of supercritical fluids are considerably higher and diffusivities considerably lower than in gases. Moreover, densities of supercritical fluids can be 100–1000 times greater than those of gases. Advantages of supercritical fluids over liquid phases rest with improved mass transfer processes due to lower fluid viscosities and higher analyte diffusivities, while advantages over gas phases rest with increased molecular interactions due to higher densities.

Other characteristics of supercritical fluids that are important to consider include the operational temperature and pressure range. Table 1.1 provides a list of nine of the most common supercritical fluids used in extraction and chromatography along with temperature, pressure, density, and dipole moment information. These nine are chosen primarily because of the convenience of their critical temperatures and critical pressures. These temperatures and pressures

Table 1.1 Physical Parameters of Selected Supercritical Fluids

Fluid	*Dipole Moment (deby)*[a]	T_c (°C)[a]	P_c (atm)[a]	ρ_c (g/mL)[a]	ρ_{400} (g/mL)[b]	ρ_1 (g/mL)[a,c]
CO_2	0.00	31.3	72.9	0.47	0.96	0.71 (63.4 atm)
N_2O	0.17	36.5	72.5	0.45	0.94	0.91 (0°C) 0.64 (59 atm)
NH_3	1.47	132.5	112.5	0.24	0.40	0.68 (−33.7°C) 0.60 (10.5 atm)
n-C_3	0.00	196.6	33.3	0.23	0.51	0.75 (1 atm)
n-C_4	0.00	152.0	37.5	0.23	0.50	0.58 (20°C) 0.57 (2.6 atm)
SF_6	0.00	45.5	37.1	0.74	1.61	1.91 (−50°C)
Xe	0.00	16.6	58.4	1.10	2.30	3.08 (111.75°C)
CCl_2F_2	0.17	111.8	40.7	0.56	1.12	1.53 (−45.6°C) 1.30 (6.7 atm)
CHF_3	1.62	25.9	46.9	0.52	1.15	1.51 (−100°C)

[a]Taken from sources given in [1], p. 14.
[b]The density at 400 atm (ρ_{400}) and $T_r = 1.03$ was calculated from compressibility data.
[c]Measurements were made under saturated conditions if no pressure is specified or were performed at 25°C if no temperature is specified.

are low enough for use with commercial instrumentation. The polarity of the supercritical fluid, as reflected in its dipole moment and polarizability, is also of considerable importance and will be discussed in more detail in Section 3.

1.3 Relationship of Supercritical Fluid Chromatography to Liquid and Gas Chromatographies

Because the characteristics of supercritical fluids fall between those of gases and liquids, supercritical fluid chromatography is a separation method with applications intermediate between those of gas and liquid chromatography. It serves as a bridge between the two techniques. Yet, fundamental chromatographic theory applies to SFC in the same manner as to GC and LC. To compare SFC with GC and LC, it is informative to evaluate practical chromatographic parameters such as efficiency, speed of analysis, migration, and selectivity.

Although chromatography is a nonequilibrium process, efficiencies of chromatographic columns are typically reported as the number of theoretical equilibration steps that occur during a chromatographic separation. This number is called the number of theoretical plates (n); the more plates a column has the more efficient is the separation. Often the generation of high efficiencies in chromatography requires considerable time; thus, the speed of analysis is also an important consideration when comparing techniques. Table 1.2 compares efficiency and analysis time ranges for various chromatographic techniques.

Table 1.2 Efficiency and Analysis Time Ranges for Various Chromatographic Techniques[a]

Technique[b]	*Velocity Range* (cm/s)	*Efficiency Range* (n)	*Efficiency/ Time Range* (n/s)	*Elution Time Range*[c] (min)	*Practical Analysis Time Range*[d] (min)
LC (packed)	0.1–0.4	5,300–8,500	14–35	2.5–10	0.5–60
SFC (packed)					
Low density	0.5–1.5	3,300–3,700	31–79	0.7–2	0.3–30
High density	0.5–1.5	3,500–5,100	42–83	0.7–2	0.3–30
SFC (open tubular)					
Low density	0.5–4	50,000–221,000	18–33	25–200	5–90
High density	0.5–4	20,000–137,000	11–13	25–200	5–90
GC (open tubular)	15–50	64,000–112,000	93–180	6–20	1.5–60

[a]Taken from [1], p. 27.

[b]LC (packed): 10-cm column length with 5-μm packing. SFC (packed): 10-cm column length with 5-μm packing. SFC (open tubular): 10-m column length with 50-μm i.d. GC (open tubular): 30-m column length with 300-μm i.d. All except the last column are calculated for nonprogrammed elution with $k = 5$.

[c]Nonprogrammed conditions, for a solute with $k = 5$.

[d]Typical programmed conditions.

While efficiency and analysis time are primarily a function of the viscosity of the mobile phase and the diffusivity of the analyte in the mobile phase, migration and selectivity are more a function of the volatility and solubility of the analyte. The more time the analyte spends in the stationary phase, the longer it will take to migrate through the column. Selectivity is a relative measure of the times two analytes spend in the stationary phase. Thus, in all forms of chromatography the affinity of the stationary phase for the analyte is a critical parameter in separation and selectivity. In addition, analytes that are more volatile (in GC) or more soluble in the mobile phase (in LC) will spend less time in the stationary phase and will migrate through the column faster. In SFC, both volatility and solubility in the mobile phase are important parameters. Thus, temperature, mobile phase density, and mobile phase composition are important parameters for controlling migration in SFC.

2 COLUMNS

The column is the heart of SFC, as it is in all forms of column chromatography. Both packed and open tubular columns can be used with their respective advantages and disadvantages. The following sections describe both theoretical and practical column considerations.

2.1 Column Efficiency

The expanded form of the Golay equation for open tubular columns is given by

$$h = \frac{2D_m}{u} + \frac{d_c^2(1 + 6k + 11k^2)u}{96(1 + k)^2 D_m} + \frac{2kd_f^2 u}{3(1 + k)^2 D_s} \tag{2}$$

where h is the plate height, u is the average mobile phase linear velocity along the column, d_c is the column internal diameter, k is the capacity factor, d_f is the stationary phase film thickness, and D_m and D_s are the solute diffusion coefficients in the mobile and stationary phases, respectively.

Figure 1.2 shows calculated van Deemter curves for open tubular columns with internal diameters from 25 to 100 μm [2]. Since both pressure and stationary phase dimensions were held constant, the k values increased in Figure 1.2 with decreasing column diameter. The D_m (CO_2 mobile phase at 40°C and 72 atm) and D_s values were assumed to be 2×10^{-4} cm^2/s and 1×10^{-6} cm^2/s, respectively. These conditions give a mobile phase density of 0.22 g/mL.

Table 1.3 lists the experimental performance data for the four different diameter columns studied [1] at a $k = 3$. Linear velocities between 5 and 10 u_{opt} are generally used in practice to reduce the analysis time. Also, from the point of view of analysis time, it is easy to see why 50- and 25-μm i.d. columns, and columns less than 15 m in length, are preferred.

So far, the discussion of column efficiency has been limited to low density supercritical fluid conditions, where diffusion coefficients are largest and

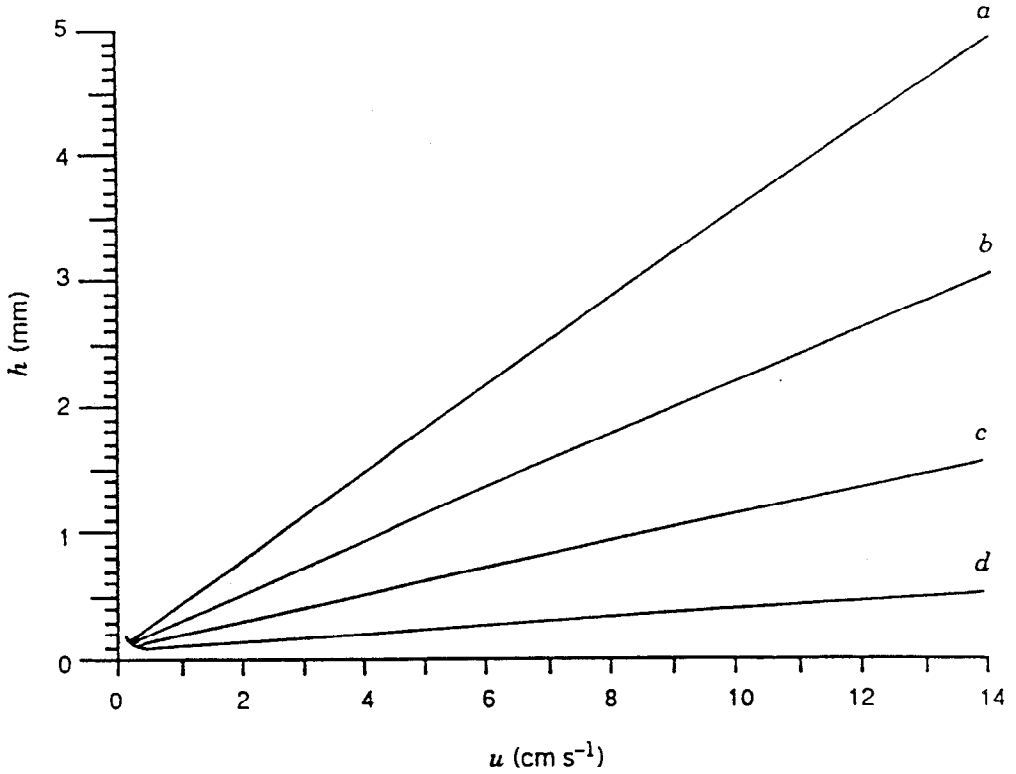

Figure 1.2 The SFC van Deemter plots for n-C_{12} on (*a*) 100-μm i.d. ($k = 2.24$), (*b*) 75-μm i.d. ($k = 2.72$), (*c*) 50-μm i.d. ($k = 3.90$), and (*d*) 25-μm i.d. ($k = 11.36$) open tubular columns. Conditions: CO_2; 40°C; 72 atm. Reprinted with permission from S. M. Fields, R. C. Kong, J. C. Fjeldsted, M. L. Lee, and P. A. Peaden, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 7, 312 (1984).

efficiencies are highest. At higher densities, the results are not as favorable. Two factors must be considered when evaluating column efficiencies at increasing densities. The first is the effect of density alone; as the density increases, D_m decreases and h increases at u larger than u_{opt}. The second factor is the inherently lower D_m values characteristic of larger solute molecules that are eluted at the

Table 1.3 Practical Open Tubular Column Efficiencies at $10\,u_{opt}$ for Different Column Diameters at $k = 3$[a]

d_c (μm)	L (m)	$10\,u_{opt}$ (cm/s)	h (mm)	n	n/m	t_R (min)	n/min
100	24	1.1	0.44	5×10^4	2300	145	370
75	24	1.4	0.30	8×10^4	3300	114	700
50	23	2.0	0.22	1×10^5	4400	77	1300
25	7[b]	4.3	0.18	4×10^4	5600	11	3500

[a]Taken from [1], p. 45.
[b]Length was shorter because of pressure drop.

higher densities. Both factors were considered in the calculation of the van Deemter curves shown in Figure 1.3.

If one were to control the linear velocity at 2 cm/s during density programming, efficiencies would decrease by nearly 75% from low-to-high density. For very large compounds, diffusivities would be even lower; for a D_m of 3×10^{-5} cm^2/s, the column efficiency would drop to about 700–800 plates per meter. From these theoretical predictions, it is obvious that significant losses in efficiency could occur at high density in open tubular column SFC. This arises because the slope of the van Deemter curve becomes very steep at high density, and the practical operating linear velocities become greater than or equal to $10\,u_{opt}$ with density programming.

There are two possible solutions to mitigate the loss of efficiency at higher density: (1) decrease the column diameter and (2) increase the operating temperature. While excellent results have been obtained using 25-μm i.d. columns, more immediate results have been obtained by increasing the temperature [3]. At constant density, an increase in temperature can result in three favorable effects: (1) an increase in solute diffusion coefficient; (2) an increase in solubility; and (3) an increase in solute volatility, with the last two effects leading to a corresponding decrease in retention (i.e., solutes elute at lower densities). It should be pointed out here that most SFC separations performed today using CO_2 are carried out at temperatures near or at 100°C.

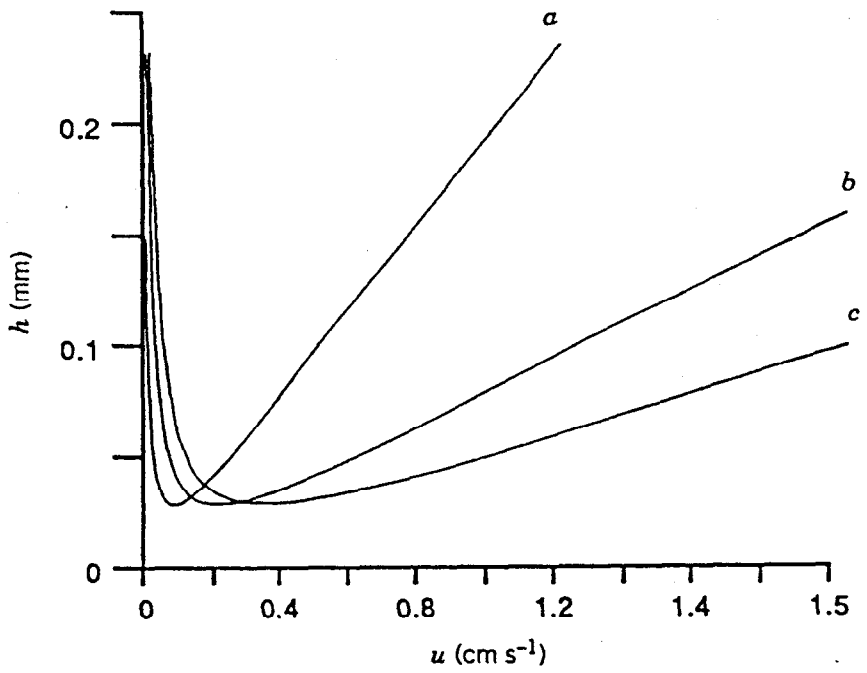

Figure 1.3 The SFC van Deemter plots for three compounds with D_m values of (*a*) 0.79 g/mL, (*b*) 0.45 g/mL, and (*c*) 0.28 g/mL. Conditions: 50-μm i.d. open tubular column; CO_2; 40°C. Data taken from S. M. Fields and M. L. Lee, *J. Chromatogr.*, **349**, 305 (1985).

One final consideration is that mobile phase linear velocity usually changes during density programming. When using a fixed restrictor, which is the usual case in open tubular column SFC, the linear velocity can more than double when programming from low-to-high density. When a 7-m × 50-μm i.d. open tubular column was programmed from 88 atm (0.47 g/mL) to 400 atm (0.96 g/mL) at 40°C, the linear velocity changed [4] from 1.3 to 10.2 cm/s. Here, much greater than 10 times the optimum velocity was produced, and the mass transfer term clearly dominated column efficiency. It should be noted that the linear velocity increased only 2.5 times for the same pressure range at the more practical temperature of 100°C.

The relationship between h and u for a packed column is more complex [5], and several expressions have been reported. Schwartz and co-workers [6] selected the Horvath–Lin equation [7] because it incorporates the resistance to mass transfer in the interior of the porous stationary phase particles. A simplification of the Horvath–Lin equation is given by

$$h = 1.5d_p + \frac{1.4D_m}{u} + \frac{2(k_0 + k + k_0k)^2 d_p^2 u}{15(1 + k_0)^2(1 + k)^2 D_m} \tag{3}$$

where d_p is the particle diameter and k_0 is the ratio of particle pore volume to particle interstitial volume in the column. The other parameters are as defined for (2). The parameter k_0 typically has a value around 0.5 for most column packings.

Two ways to look at column efficiency are from the viewpoint of plate height (h) or total number of theoretical plates (n). The smallest plate heights are obtained by introducing packing material to form packed columns. This minimizes the contribution to the plate height from the resistance to mass transfer in the mobile phase. However, the packing also reduces the permeability of the column and creates a resistance to flow, such that the length of the column must usually be limited to less than 25 cm. Table 1.4 gives a comparison of total plates calculated for both packed and open tubular columns under practical conditions at 100°C. The efficiencies at low density were calculated for practical initial linear velocities: approximately $5u_{opt}$ ($u_{opt} = 0.5$ cm/s) for the open tubular column, and $0.2\,u_{opt}$ ($u_{opt} = 3.0$ cm/s) for the packed column, which is close to the practical u_{opt} when 5-μm particles are used [8]. Practical u_{opt} values are known to differ from theoretical u_{opt} for packed columns, especially for small particle sizes. As density increases in the packed column during density programming, the linear velocity should move to a more favorable value of $1.3\,u_{opt}$ ($u_{opt} = 1.6$ cm/s) with a concomitant increase in efficiency; however, these efficiencies are never realized in practice. The open tubular column, on the other hand, starts out with extremely high efficiency at a practical velocity of 5 times the theoretical u_{opt}, but the dramatic increase in linear velocity that is achieved at the final density corresponds to $28\,u_{opt}$ ($u_{opt} = 0.2$ cm/s), giving a 5.4 times reduction in efficiency.

Table 1.4 Calculated[a] Total Theoretical Plates for Packed and Open Tubular Columns Under Practical Conditions

			n		u (cm/s)	
Column Type	d_p, d_c (μm)	*Length* (m)	*Low* Density[b]	*High Density*[c]	*Low Density*[b]	*High Density*[c]
Packed	5	0.1	5,200	9,100	0.6 ($0.2\,u_{opt}$)	2.1 ($1.3\,u_{opt}$)
Open tubular	50	10	102,000	19,000	2.5 ($5.0\,u_{opt}$)	5.8 ($2.8\,u_{opt}$)

[a]Plate heights were calculated from (2) and (3) for open tubular and packed columns, respectively. The following conditions were assumed: *Open tubular column:* $D_s = 1 \times 10^{-6}$ cm^2/s, $d_f = 0.25$ μm, and $k = 2$. *Packed column:* $k_0 = 0.5$, $d_p = 5$ μm, and $k = 2$.
[b]Low density conditions were assumed for the CO_2 mobile phase at 100°C, 100 atm, 0.19 g/mL, and $D_m = 5 \times 10^{-4}$ cm^2/s.
[c]High density conditions were assumed for the CO_2 mobile phase at 100°C, 400 atm, 0.76 g/mL, and $D_m = 2 \times 10^{-4}$ cm^2/s.

2.2 Column Pressure Drop

The pressure drop is important in SFC because density and, hence, solvating power is a direct result of the pressure. If there is a pressure drop, there is also a density drop, and the solubilities of solutes decrease along the length of the column. This finding is especially important in considering the type of column to use; typical open tubular columns have minimal pressure drops, while significant drops are usually associated with packed columns. In most cases, pressure drop effects will be insignificant for open tubular columns [9].

2.3 Speed of Analysis

The analysis time in chromatography for a given solute can be calculated from

$$t_R = \frac{L}{u}(1 + k) \tag{4}$$

where the terms are as previously defined. When fast analysis times are sought, higher than optimum mobile phase linear velocities are used.

In making a direct comparison of the speed of analysis for packed and open tubular columns, it must be assumed that both columns can produce a sufficient number of total theoretical plates to accomplish the desired separation. Speed is of no value if resolution is not satisfied. If n is set equal for both columns at 5000 total plates, the analysis times can be calculated for both low and high densities from (4). For the conditions described in Table 1.4, it was found that a

1-m × 50-μm i.d. open tubular column could give an analysis time of 2.0 compared to 0.8 min using a 10-cm packed column with 5-μm particles at low density. While the open tubular column can approach the speed of the packed column at lower densities, the differences are more pronounced at higher densities.

2.4 Sample Capacity and Loadability

Unlike open tubular column chromatography, the packed column internal diameter does not, in general, affect the separation efficiency, and it can be increased to increase sample capacity and loadability. The sample capacity refers to the maximum amount of sample solute that can be introduced on the column without causing significant fronting of the chromatographic peak. On the other hand, sample loadability, as defined here, refers to the maximum volume of sample (solutes plus solvent) that can be introduced on the column without causing significant degradation of column efficiency.

For open tubular columns, an equation has been derived [10] to estimate allowable injection volumes based on column diameter and length, plate height, capacity factor, and acceptable resolution loss

$$V_i = 0.866\,\pi d_c^2 (Lh)^{1/2} \left[\frac{1}{(1-\Delta R_s)^2} - 1\right]^{1/2} (1+k) \tag{5}$$

where V_i is the injection volume, ΔR_s is the fractional resolution loss, and the other variables are as previously defined. For a 10-m × 50-μm i.d. column under practical operating conditions ($u = 10\,u_{opt}$ and $k = 2$), and for a 1% allowable resolution loss, the maximum sample volume that can be injected is 96 nL. Obviously in practice, methods for sample volume reduction and solute focusing must be used for sample introduction into open tubular columns, especially when smaller diameter columns are used.

The sample volume loadability (L) of a sample component on a chromatographic column has been defined as

$$L = c_{m,max} V \tag{6}$$

where $c_{m,max}$ is the maximum concentration of a particular component that can be accommodated by the mobile phase without causing more than 5% deviation from the linear distribution isotherm of the phase system for a particular injection volume (V) [6]. Obviously, the loadability of packed columns is much higher than that of open tubular columns.

Stationary phase film thicknesses of up to 1 μm can be used in 50-μm i.d. open tubular columns for SFC with only minimal losses in chromatographic efficiency. Based on a definition of sample capacity as the amount injected for which it takes twice the length of time for the leading edge of the chromatographic peak as for the trailing edge, practical measurements have lead to a sample capacity of 100 ng (alkanes) on a 10-m × 50-μm i.d. column coated with a

0.25-μm film of a polymethylsiloxane stationary phase. A 1-μm film should increase this capacity to approximately 400 ng with less than a 10% resolution loss.

2.5 General Requirements for Column Technology

The column dimensions used in SFC are tabulated in Table 1.5. The traditional names that have been adopted for the different packed column types are borrowed from LC. The conventional 4–6-mm i.d. packed column is not the most common dimension of packed columns in SFC as it is in LC. Limitations in volumetric pumping speeds and/or volumetric flow rates for the detectors of choice have helped to make packed columns of micro sizes increasingly common in SFC. The packed capillary column has the advantages of lower volumetric flow rates, making them more compatible with mass flow sensitive detectors; smaller elution peak volumes, which provide greater sensitivity in concentration sensitive detectors; easier sample transfer in multidimensional systems; and higher permeability, which allows the use of longer columns for better resolution. Open tubular columns for SFC are typically 3–10-m × 50-μm i.d., which represents a practical compromise in efficiency and speed.

The requirements that must be satisfied by column technology include (1) well-deactivated surfaces and stationary phases, (2) uniform stationary phase films, and (3) well-immobilized stationary phase films. There continues to be a demand for more inert columns, and this demand has resulted in innovations in deactivation procedures for column and support materials. The most deactivated columns have been open tubular columns. This is a result of their low surface area and much lower porosity when compared to silica or alumina packing materials. In addition to causing adsorption of polar analytes, active sites lead to poorly controlled and nonreproducible retention. Active sites generally necessitate the use of polar modifiers.

In the packed column, the stationary phase is normally near monomolecular thickness and is polymerized and chemically bonded to the support. Immobilization (generally cross-linking of the polymeric phase) is an essential ingredient in the preparation of open tubular columns. It must be performed to resist dissolution, but without lowering solute diffusion within the phase.

Table 1.5 Typical Column Dimensions

Column Type	*Internal Diameter* (mm)	*Length* (m)
Conventional packed	2–4.6	0.03–0.25
Microbore packed	0.5–2	0.03–0.25
Packed capillary	0.1–0.5	0.05–0.5
Open tubular	0.025–0.1	1–35

2.6 Packed Column Technology

Since the early days of SFC, packed column SFC technology has depended on materials available from the current state-of-the-art LC technology. It is not surprising that LC packing materials perform well under SFC conditions, since both techniques depend on the ability of the mobile phase to solvate analyte molecules.

Particle sizes referred to in publications normally vary from 3 to 10 μm in diameter with pore sizes ranging from 100 to 300 Å (corresponding to a surface area of ca. 100–300 m^2/g). Of these, the most commonly used particle size is 5-μm diameter. This particular size is popular because it is small enough to give relatively small plate heights, while being commercially available in sufficient uniformity and narrow distribution to allow efficient packing to be accomplished. Smaller particles provide smaller plate heights; however, they also reduce permeability and increase the pressure drop across the column. The feasibility of working with small diameter particles in SFC has been discussed by several groups [11–15].

2.7 Open Tubular Column Technology

Open tubular columns for SFC must possess the usual qualities of high efficiency, inertness, and lasting stability, which are characteristic of open tubular columns for GC. The main differences in the preparation of the columns are related to the smaller internal diameters characteristic of SFC columns. These are discussed in detail in [1].

2.8 Stationary Phases for Supercritical Fluid Chromatography

The stationary phase plays an important role in achieving high performance in SFC. Many stationary phases developed for either LC or GC can be adopted for use in SFC. This includes phases exhibiting all types of solute–stationary phase interactions and selectivities, such as adsorption, dispersion, dipole-induced dipole, dipole–dipole, and size and shape, as well as combinations of these interactions.

The packed columns used today in SFC are usually columns developed for LC. Up to an order of magnitude greater resolution per unit time is achieved by simply changing from a liquid to a supercritical mobile phase.

There is a fine line between the effects of the stationary phase support and the stationary phase in packed columns, since they both usually contribute to the retention mechanism. Furthermore, the mobile phase and/or mobile phase modifiers interact with the stationary phase to form a modified surface. This final surface should be considered as the real stationary phase.

Adsorbents, such as silica and alumina, have been used extensively as stationary phases in the past. These phases are useful for nonpolar compounds; however, they lead to both reversible and irreversible adsorption of polar solutes in SFC, especially when neat CO_2 is used as the mobile phase. The limited

success experienced to date in achieving a high level of deactivation of these materials suggests their rather limited future potential.

Modification of the typical small particle size silicas and aluminas with bonded stationary phases such as octyl, octadecyl, cyanoalkyl, aminoalkyl, and diolalkyl provide less adsorptive packing materials and a wide range of polarities for dipole–dipole and dipole-induced dipole interactions. In most cases, except for the most nonpolar molecules, polar organic modifiers are required for elution of analytes from these materials.

Most commercial phases are monomeric in nature because they produce a monolayer coverage of phase on the solid support. Excess silanol groups in this monolayer may be either end-capped, used to induce polymerization within this monolayer, or they may be left to take part in selective interactions as part of the stationary phase.

Polysiloxanes are extensively used as polymeric backbones in stationary phases for open tubular columns. The chemical and physical stabilities of the polysiloxanes, along with the desirable flexibility of the Si—O bond that leads to good diffusion of sample analytes, make them ideal as stationary phases. Polysiloxanes have been substituted with a wide range of chemical groups for selective interactions with different types of samples.

Dispersion interactions are commonly used in open tubular column SFC. The great inertness and efficiency of columns coated with polymethylsiloxanes are utilized in SFC, but enhanced partitioning was demonstrated using *n*-octyl-substituted polysiloxanes compared to methyl substituted phases [16]. This *n*-octyl phase also has a sufficient density of C—C bonds such that these columns could be used for a limited time with neat NH_3 as the mobile phase [17].

The biphenyl phase with 30 mol% substitution is usually preferred over the 50% phenyl phase because the larger, more polarizable biphenyl group provides greater interaction with the analytes [18]. In addition, the biphenyl phase contains a higher percentage of methyl groups than the corresponding 50% phenyl phase and is therefore easier to immobilize on the column wall. Analytes containing either electron-donating or electron-withdrawing groups can induce polarity in the biphenyl stationary phase. The lack of polar interactions makes this phase ideal for the separation of closely related polar solutes without excessive retention.

The most widely used polar stationary phases in open tubular column SFC are the cyanopropyl polysiloxanes [19]. With CO_2 as the mobile phase, these stationary phases have been particularly useful for the analysis of compounds containing carboxylic acid functional groups.

A highly ordered liquid crystalline polysiloxane stationary phase was reported by Chang and co-workers [20, 21] for use in SFC. A dramatic enhancement in resolution over GC was demonstrated for selected geometrical isomers. The SFC elution was performed at 120°C, where the stationary phase was more ordered than at the 230°C elution temperature in GC.

Chiral separations in SFC to date have been primarily explored using packed column technology developed for LC analysis. A thermally stable chiral amide

phase developed for GC [22] was found to give higher resolution in SFC than in GC for some derivatized amino acids. The gain in selectivity at the lower elution temperature more than compensated for the loss in efficiency from the lower diffusion in the supercritical fluid.

3 MOBILE PHASES

The mobile phase in SFC is the most influential parameter governing solute retention on the column. Unlike in GC, where the mobile phase is relatively inert, SFC mobile phases play an active role in altering the distribution coefficient of the solute between the stationary phase and a compressed carrier fluid phase. The mobile phase chosen in SFC is often selected with respect to its departure from ideal gas behavior, a characteristic that allows its densification through the application of external pressure. Supercritical fluid chromatography also differs from LC where solute retention is usually adjusted by changing either the chemical nature of the mobile or stationary phase within the column. Only at very high applied pressures does one observe significant changes in LC retention parameters [23].

3.1 General Characteristics

Fluid density is the key parameter for understanding the behavior of supercritical fluids. Since density is a function of both pressure and temperature, the effects of these two variables can best be understood by using a corresponding states plot, in which the reduced density is expressed as a function of reduced temperature and pressure. The critical point of a fluid occurs when the above physical properties (pressure, temperature, and density) are all equal to their critical values', hence, the reduced pressure, temperature, and density will all be equal to unity. This corresponds to the apex of the gas–liquid region as shown on the plot of reduced state in Figure 1.4.

Supercritical fluid chromatography is performed above the critical temperature of the fluid, that is, above the isotherm equal to unity. Reduced pressures ranging from 0.6 to values in excess of 20 have been reported for SFC. This range of pressure and temperature results in reduced fluid densities ranging from 0.3 to values in excess of 2.0. Inspection of Figure 1.4 reveals that supercritical fluids under high pressures will approach reduced densities that are similar to those exhibited by the liquid state (2.5–3.0). The shaded regions in Figure 1.4 are typical operating conditions that have been reported for SFC. The choice of these conditions is largely mandated by the desire to affect the largest change in fluid density commensurate with performing SFC at a low temperature. This is accomplished by operating close to the critical temperature (T_c) of the fluid and in the region of the eluent's critical pressure (P_c).

It is obvious from this discussion that the mobile phase in SFC can take on a range of densities intermediate between those encountered in gas or liquid

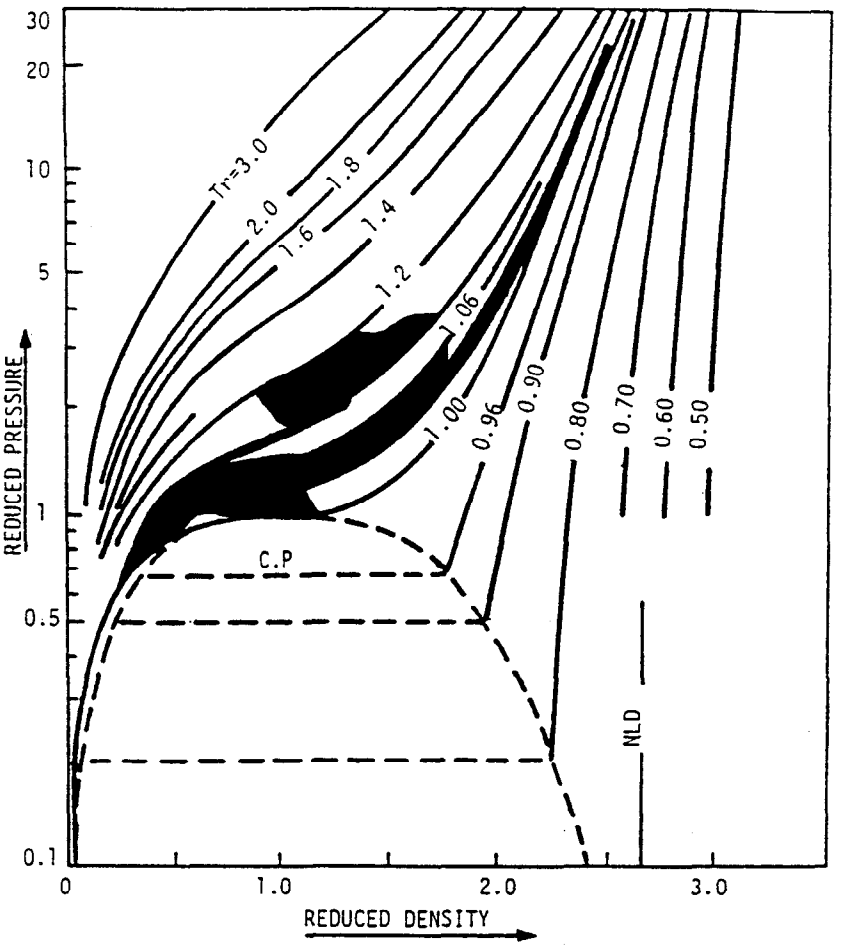

Figure 1.4 Reduced state plot showing application range for supercritical fluid chromatography. Reprinted with permission from J. C. Giddings, M. N. Myers, L. McLaren, and R. A. Keller, *Science*, **162**, 67 (1968). Copyright © 1968 by the AAAS.

chromatography. Of equal importance to the chromatographer are the superior mass transfer characteristics exhibited by supercritical fluids. For example, the diffusivity of supercritical CO_2 is approximately two orders of magnitude greater than those exhibited by liquid solvents. Similarly, the viscosity of supercritical CO_2 is at least 20 times larger than the viscosities associated with liquid media. These physical properties influence the theoretical plate heights that are obtainable with SFC and result in a smaller nonequilibrium contribution to peak broadening in SFC relative to that found for LC methods.

The variation in fundamental SFC retention parameters, such as the capacity factor k, with pressure has been found to be nonlinear. Over a select operating range, fluid density has been shown to be universely proportional to the capacity factor [24]. More sophisticated correlations have been proposed to describe the effect of mobile phase density on solute retention in SFC [25, 26]. A "unified theory" has recently been offered by Martire [27] to predict retention behavior in GC, SFC, and LC. Attempts to relate retention in SFC to solute properties, such as solubility parameters, are at best difficult, due to the complex interaction of the mobile phase with the column stationary phase [28]. The solubility

enhancement of the solute in the supercritical fluid phase can be predicted by standard thermodynamic methods; however, it must be realized that this is only one factor that governs retention in SFC.

In Section 3 we shall examine in detail how the mobile phase influences separation in SFC. Both neat fluids, and mobile phases containing cosolvents or specific additives will be described. Finally, the various programming modes will be examined in terms of the potential they afford the analyst in performing separations by SFC.

3.2 Neat Fluids

The SFC mobile phases employing a single fluid have been used extensively since the inception of the technique in 1962. The properties of these mobile phases can best be understood by examining the critical parameters associated with each fluid. Table 1.6 [29] lists the critical temperature, pressure, density, and volume for 31 fluids, many of which have been utilized in SFC. By far the most popular fluid for SFC has been CO_2, because of its low critical temperature, relative inertness, low toxicity, and nonflammability; CO_2 and its isoelectronic analog, N_2O, also exhibit a high degree of nonideality, which permits the generation of high fluid densities when the gases are compressed.

Other polyatomic gases, such as ammonia and sulfur dioxide have seen limited use due to their high critical pressures and corrosive nature. Sulfur hexafluoride and xenon have been used sparingly due to their high cost. Fluorocarbon gases have also been used as eluents [30] due to their low critical temperatures and unique selectivity for certain solutes [31]. The hydrocarbon fluids listed in Table 1.6 have seen limited use due to their high flammability; however, *n*-pentane has been utilized as a mobile phase for the separation of oligomers [32]. Polar organic moieties, such as isopropyl or diethyl ether, have seen limited use due to their high T_c; however, selected applications have been reported for the separation of polynuclear aromatics [33] and polymers [34].

The solvent power of neat fluids has been rationalized by Giddings and coworkers [35, 36] in terms of the solubility parameter concept. Here, the solubility parameter δ of the fluid is given by

$$\delta = 1.25\, P_c^{1/2}(\rho_r/\rho_{r,1}) \tag{7}$$

where ρ_r and $\rho_{r,1}$ are reduced densities of the gas and the fluid in the quasiliquid state, respectively. Calculation of the solubility parameter as a function of fluid density permits a comparison to conventional liquid solubility parameter values [37] as well as the correlation of solute solubilities in supercritical fluid media [38].

In Figure 1.5, solubility parameters have been calculated for nitrous oxide as a function of temperature and pressure using literature data [39]. The solubility parameter shows a steep dependence on pressure at the critical isobar and temperature. This is due to the large increase in the reduced density of the critical fluid as one appraches its critical pressure. It is not necessary to compress

Table 1.6 Critical Parameters of Selected Fluids[a]

Fluid	T_c (°C)	P_c (atm)	ρ_c (g/mL)	V_c (mL/mol)
CO_2	31.1	72.8	0.468	94
N_2O	36.4	71.5	0.452	97
SF_6	45.5	37.0	0.738	198
SO_2	158	78	0.525	122
CS_2	279	78	0.448	170
Xe	16.6	57.6	1.113	118
H_2O	374.1	217.6	0.322	56
Methanol	239.4	79.9	0.272	118
Ethanol	243.0	63.0	0.276	167
Propanol	263.5	51.0	0.275	218
2-Propanol	235.1	47.0	0.273	220
Dimethyl ether	127	52.6	0.259	178
Methyl ethyl ether	164.7	43.4	0.272	221
Diethyl ether	193.6	36.3	0.267	280
Acetonitrile	274.7	47.7	0.237	173
NH_3	132.3	111.3	0.235	72
Methylamine	156.9	40.2	0.222	148
Dimethylamine	164.6	52.4	0.241	187
Trimethylamine	160.1	40.2	0.233	254
Triethylamine	258.9	30	0.259	390
Ethane	32.4	48.3	0.203	140
Ethylene	10.0	51.2	0.227	124
Propane	96.8	42.0	0.220	203
n-Butane	152.0	37.5	0.228	255
n-Petane	196.6	41.7	0.554	304
Benzene	288.9	48.3	0.302	259
Trifluoromethane	25.9	47.7	0.516	136
Tetrafluoromethane	−45.7	41.4	0.628	140
Chlorotrifluoromethane	28.8	38.7	0.580	180
Chlorodifluoromethane	96.0	49.1	0.524	165
Dichlorodifluoromethane	111.7	39.4	0.557	217

[a]Data taken from [29].

the mobile phase to a density that yields a solubility parameter equivalent to the solute being chromatographed. However, a large difference in the solubility parameters of the solute and the fluid may make chromatography difficult due to the immiscibility of the solute with the fluid mobile phase.

Other measures of fluid polarity have been proposed, and solvatochromic measurements [40, 41] indicate that at higher densities, the cybotatic region around the solute molecules is highly ordered. Examples of weak complexation between solute and solvent fluid have been reported in the supercritical fluid state [42, 43]. Inter- [44] and intramolecular hydrogen bonding [45] can

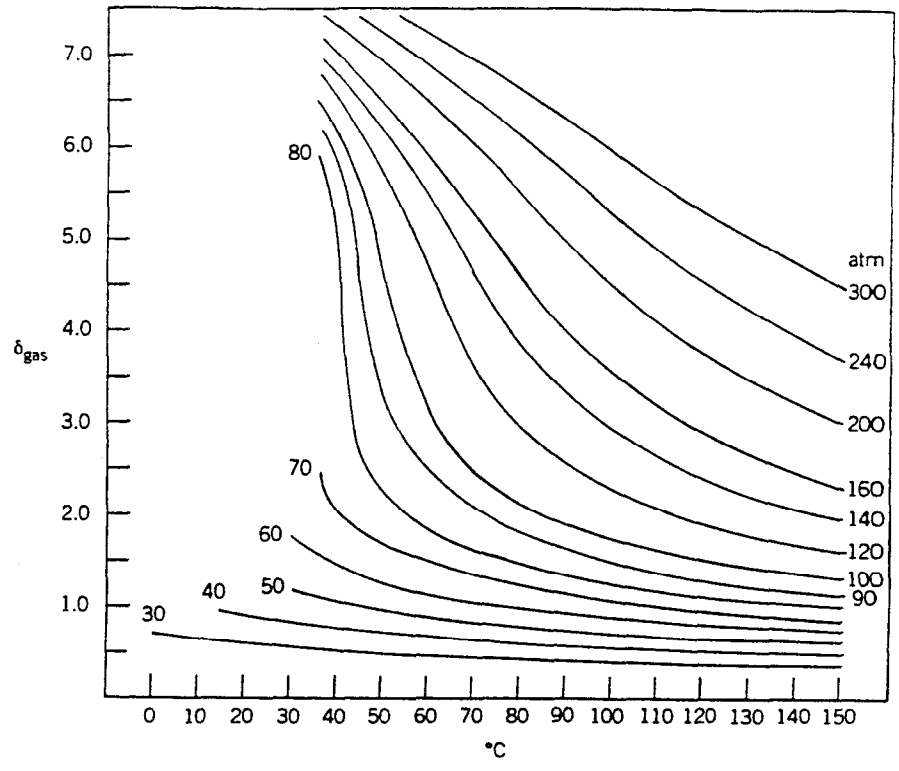

Figure 1.5 Solubility parameter for nitrous oxide as a function of temperature and pressure. Reprinted with permission from J. W. King, "Preprints—Polymeric Materials Science and Engineering," American Chemical Society, Washington, DC, 1984. Copyright © 1984 American Chemical Society.

influence retention behavior in SFC due to enhanced solubility in the mobile phase or by modification of the solute's effective molar volume. Of course the ultimate complexation, a chemical reaction between solute and supercritical fluid must be avoided to assure successful SFC. For example, reactions between primary or secondary amines and CO_2 have been reported to occur during SFC [46].

3.3 Mixed Fluids

Mixed mobile fluids have been incorporated in SFC for a number of purposes. Perhaps the most important use of mixed fluids has been the addition of a polar organic modifier to the supercritical fluid to enhance the solvent power of the eluent. This step is generally taken to enhance the solubilization of polar solutes in dense fluids or to reduce the retention volume of the analyte in the column. Table 1.7 [47, 48] lists several useful modifiers that have been utilized in SFC. Note that the addition of these solvents into a supercritical fluid phase will modify the polarity of the eluent due to the high dielectric constants or polarity indexes associated with the organic modifiers. The polarity indexes in Table 1.7 are derived from the scheme proposed by Snyder [49] in which the overall polarity index is the sum of contributions due to each type of solute–solvent

Table 1.7 Frequently Used Modifiers in Supercritical Fluid Chromatography

Modifier	T_c (°C)	P_c (atm)	*Molecular Mass*	*Dielectric Constant*[a] *at 20°C*	*Polarity Index*[a]
Methanol	239.4	79.9	32.04	32.70	5.1
Ethanol	243.0	63.0	46.07	24.3	4.3
1-Propanol	263.5	51.0	60.10	20.33	4.0
2-Propanol	235.1	47.0	60.10	20.33	4.0
1-Hexanol	336.8	40.0	102.18	13.3	3.5
2-Methoxy ethanol	302	52.2	76.10	16.93	5.5
Tetrahydrofuran	267.0	51.2	72.11	7.58	4.0
1,4-Dioxane	314	51.4	88.11	2.25	4.8
Acetonitrile	275	47.7	41.05	37.5	5.8
Dichloromethane	237	60.0	84.93	8.93[b]	
Chloroform	263.2	54.2	119.38	4.81	4.1
Propylene carbonate	352.0		102.09	69.0	6.1
N,*N*-Dimethylacetamide	384		87.12	37.78[b]	6.5
Dimethyl sulfoxide	465.0		78.13	46.68	7.2
Formic acid	307		46.02	58.5[c]	
Water	374.1	217.6	18.01	80.1	10.2
Carbon disulfide	279	78.0	76.13	2.64[c]	

[a]Data taken from [47].
[b]At 25°C.
[c]Data taken from [48].

interaction. Randall [50, 51] used this concept as a basis for choosing a modifier in SFC where CO_2 is employed as the mobile phase. In these studies, it was shown that the chromatographic capacity factors and relative separation factors were affected not only by the modifier identity, but also by the concentration of the modifier in the mixed fluid eluent.

The modifiers listed in Table 1.7 have quite different critical temperatures and pressures. These data suggest that caution must be taken when using mixed fluids to assure that the components are miscible over the range of temperatures and pressures that are used. These conditions can be established by using thermodynamic data or by making precise phase equilibrium measurements. Calculation of pseudocritical constants for mixed mobile phases have been approximated by the method of Kay [52]. Alternatively, useful compendiums of actual vapor–liquid equilibria at high pressure exist, which define conditions for the existence of the one phase region for such systems as CO_2 and organic cosolvents [53]. Recently, a laser light scattering method has been utilized to determine phase transitions of mixed mobile phases in the critical region [54].

Frequently, modifiers are added to the supercritical fluid eluent to eliminate adsorptive effects exhibited by solutes in packed column SFC. In this case, the modifier eliminates the strong interaction between adsorptive sites and the polar

solute resulting in symmetrical peak profiles. The dramatic results that can be produced by the inclusion of such modifiers in SFC are shown in Figure 1.6 for the separation azo-dyes on a column packed with diol-modified silica [55]. In this case, the peak shape is improved and both the order of elution and resolution between the component peaks are affected by the choice of modifier. Similarly, water has been used as modifier in CO_2 to improve the symmetry of fatty acid peaks eluting from columns packed with bonded silica stationary phases [56].

Mixed fluids may also incorporate special additives that can affect both the solubilization of the solute in the fluid phase or enhance solute elution through the chromatographic column. Such additives, because of their extremely polar nature, may have limited solubility in common SFC mobile phases. These

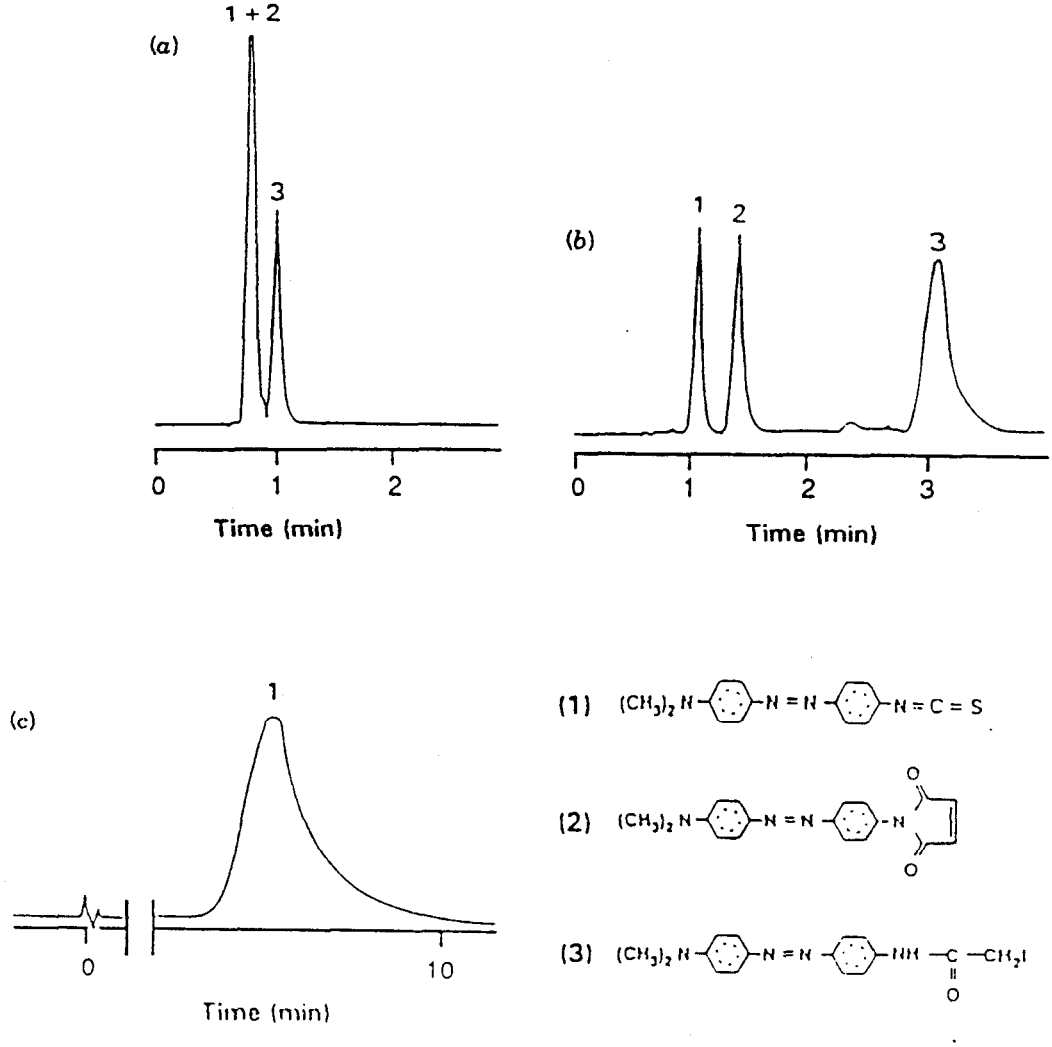

Figure 1.6 The SFC chromatograms of azo dyes showing the effect of modifier in CO_2 with (*A*) 6 mol% propylene carbonate, (*B*) 6 mol% *N,N*-dimethylacetamide, compared to (*C*) without modifier. Conditions: 25-cm × 4.6-mm i.d. packed column, diol-modified silica, 5-μm particles; CO_2; 60°C; 370 atm; (420 nm). Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, A Division of Preston Industries, Inc., Niles, IL.

compounds can be solubilized in the supercritical fluid eluent by dissolving them in a suitable modifier, thereby making the mobile phase a ternary system. An excellent example of this principle is the use of citric and trifluoroacetic acids in methanol–carbon dioxide mobile phases to affect the capacity factors and peak shapes of polar aromatic acids eluting from packed silica SFC columns [57, 58]. Similarly, polar ionic solutes can be chromatographed using nonpolar supercritical fluid eluents, such as ethane, by incorporating reverse micelles in the mobile phase [59].

3.4 Programming Techniques

An attractive feature of SFC is the capability of changing the mobile phase parameters of pressure, density, temperature, or composition with respect to time. Such programming methods have allowed the fractionation of extremely complex mixtures by SFC and have become the routine mode in which SFC is utilized in the analytical laboratory. A comprehensive review of "SFC gradient methods" has been provided by Klesper and Schmitz [60]; therefore, we shall only cover the salient points associated with various programming methods in this section. Table 1.8 tabulates the various programming techniques that are possible with SFC [60]. By far, the most prevalent techniques that have been used in the laboratory are either pressure or density programming at constant temperature and an isocratic mobile phase composition. We shall discuss these

Table 1.8 Gradients in Supercritical Fluid Chromatography[a]

Simple Gradients

(a) Temperature
(b) Pressure/density
(c) Velocity
(d) Eluent composition

Isocratic Multiple Gradients

(a) Pressure/density—temperature
(b) Pressure/density—velocity
(c) Temperature—velocity
(d) Pressure/density—temperature—velocity

Nonisocratic Multiple Gradients

(e) Eluent composition—pressure/density
(f) Eluent composition—temperature
(g) Eluent composition—velocity
(h) Ternary and quaternary gradients (containing an eluent composition gradient, e.g., "eluent composition-pressure/density-temperature")

[a]Reprinted with permission from E. Klesper and F. P. Schmitz, J. Supercrit. Fluids, 1, 45 (1988).

two programming techniques and the effect of varying the temperature of the mobile phase in SFC below.

The variation in the capacity factor with pressure has been shown by van Wasen [61] to be directly proportional to the difference in the partial molar volumes of the solute in the mobile and stationary phases, respectively. The separation of solutes in programmed density or pressure SFC is also dependent on the pressure range required for initially solubilizing the solutes in the mobile phase. The range of pressures required for achieving solute miscibility in the mobile phase can be predicted by the method of King [62]. Thus, programmed pressure or density SFC can be envisioned as a dual process, consisting of solubilization of the solute in the supercritical fluid phase (SFE) followed by a pressure dependent partition process that takes place during SFC. For this reason, programmed SFC should be carried out at the lowest possible starting density and rate of density change per unit time, so as to produce the maximum fractionation between the injected solutes.

The effect of mobile phase density on selectivity α and capacity factor k can be calculated by the equations given by Peaden and Lee [10]

$$\ln \alpha = B_0 - m\rho \tag{8}$$

and

$$\ln k = a - b\rho \tag{9}$$

where B_0, m, a, and b are constants dependent on the type of solute being separated, the nature of the mobile and stationary phase, and the temperature of the mobile phase. Since both k and α decrease with increasing density in programmed SFC, the separation efficiency will also decrease as the mobile phase density is increased. This is due in part to the decrease in the diffusion coefficients of the mobile phase and dissolved solutes, resulting in substantial peak broadening in the latter portion of the chromatogram. Consequently, the mass transfer term in the van Deemter equation tends to be larger at higher mobile phase densities than at lower densities.

Overall, programmed density or pressure SFC can be viewed as a merging of GC and LC elution behavior. In the initial portion of the pressure program, the elution of solutes is primarily controlled by solute volatility as in GC; however, as the program progresses and the mobile assumes a higher density, the elution of solutes becomes dependent on the "solvent" strength of the eluent. Hence, the latter peaks in Figure 1.7, which depicts the SFC separation of oligomers, are "solution" controlled, while the lower molecular weight oligomers ($n = 5-10$) partition into the mobile phase primarily by a volatilization mechanism. It is this ability of pressure programmed SFC to "bridge the gap" between LC and GC, that makes it such a versatile analytical technique.

Temperature can also have a significant effect on the peak resolution obtained using pressure programmed SFC. For example, the resultant chroma-

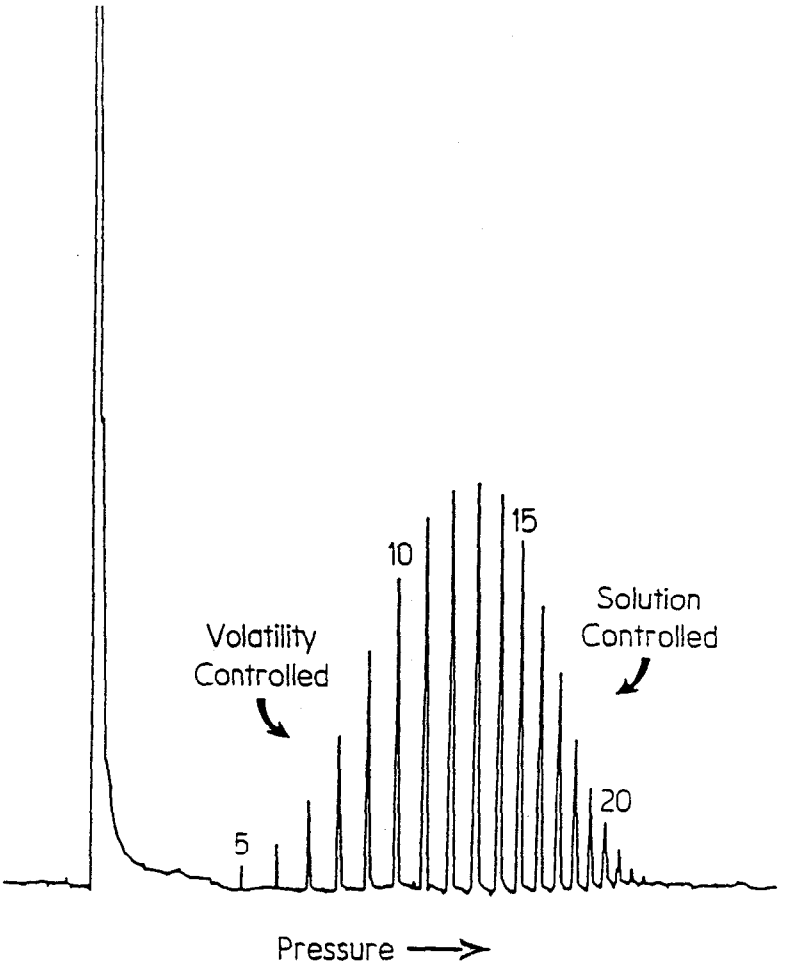

Figure 1.7 Pressure-programmed capillary supercritical fluid chromatographic separation of oligomers.

tograms for poly(styrene) oligomers shown in Figure 1.8 have been significantly affected by the temperature. The binary eluent in this case consists of a mixture of 95% *n*-pentane and 5% methanol by volume, which has a critical temperature of 202°C. In Figure 1.8, the SFC pressure programmed runs conducted at temperatures below T_c (at 180 and 190°C) have poor resolution between the oligomers. Operation close to the critical point of the mobile phase (200°C), however, yields excellent resolution of oligomers, due to the improved mass transfer of the solutes in the mobile phase.

The temperature dependence of solute retention in SFC has been rationalized by several thermodynamic approaches [63–65]. Perhaps the most instructive approach is that of Chester and Ennis [64], where trends in capacity factor with temperature have been divided into GC- and LC-like behavior, expressed by the following equation as

$$\log k = \frac{-0.43\Delta H_s}{RT} - \log \beta + \frac{0.43\Delta H_m}{RT} \tag{10}$$

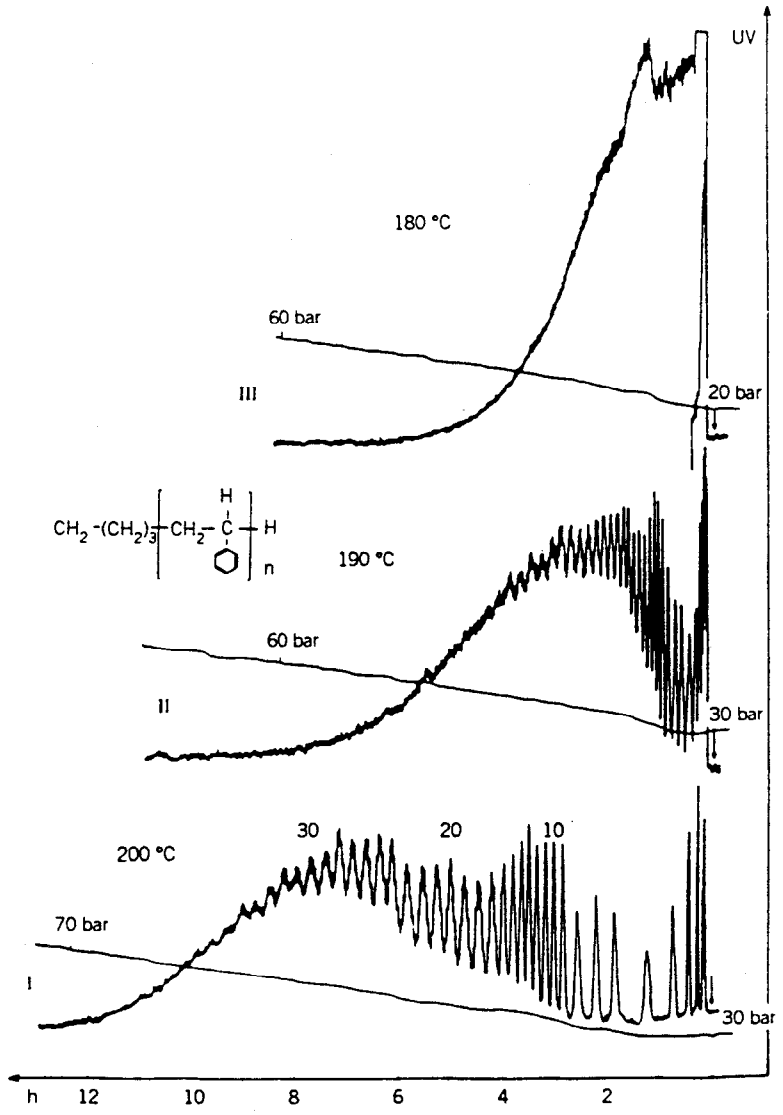

Figure 1.8 Chromatograms of poly(styrene) oligomers at different temperatures, below or near the critical temperature. Similar pressure programs at 3.3 atm/min; (180°C), 2.8 mL/min; (190°C), 3.0 mL/min; (200°C), 2.8 mL/min. Reprinted with permission from E. Klesper and W. Hartmann, *J. Polym. Sci., Polym. Lett. Ed.*, **15**, 707 (1977).

where ΔH_s is the partial molar heat of solution of the solute in the stationary phase, ΔH_m is the partial molar heat of solution of the solute in the mobile phase, and β is the column phase ratio. Figure 1.9 depicts the retention behavior in SFC for three *n*-alkanes at two pressures over an extended temperature range. Such trends are in agreement with those predicted by (10) since the initial slope of the $\ln k$ versus $1/T$ plot is equivalent in form to a van't Hoff relationship in GC; that is, the capacity factor increases with decreasing temperature. At even lower temperatures, the curves in Figure 1.9 begin to depart from linearity, due to the increasing importance of the last term in (10). Such a trend suggests that solute retention in SFC at low temperatures is governed by mobile phase solvation akin to behavior observed in LC separations.

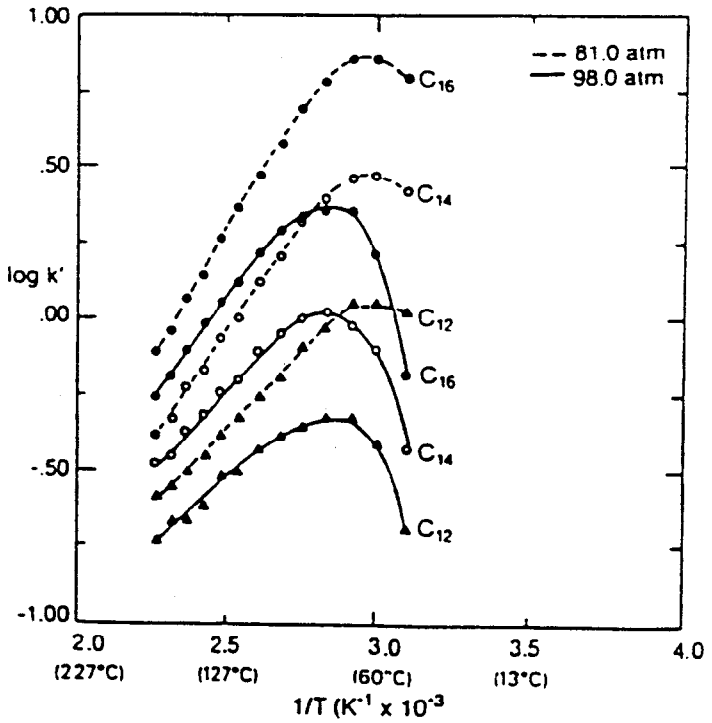

Figure 1.9 Log k versus $1/T$ for three n-hydrocarbons at constant pressures of 81 and 98 atm on a BP-10 column. Reprinted with permission from T. L. Chester and D. P. Innis, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, **8**, 561 (1985).

The above temperature dependence of solutes in SFC suggests that negative temperature programming can produce separations equivalent to those obtained by pressure programming. To date, limited use has been made of this concept, although several investigators [66, 67] produced acceptable separations using this principle. Resolution of unresolved peaks toward the end of a pressure programmed SFC run can sometimes be affected by using negative temperature programming, thereby producing in effect a multiple gradient program.

Other programming options exist, but have been applied sparingly to special separation problems. For example, asymptotic density programming as opposed to linear density programming has found use for the separation of oligomeric mixtures due to the improved resolution of higher molecular weight solutes at the end of the SFC run [68]. Similarly, compositional changes during the SFC run have afforded some unique separations of oligomers and polymers by SFC. An excellent review of this field has been written by Schmitz and Klesper [69].

3.5 Experimental Considerations

The choice of a mobile phase for SFC is often mandated by factors other than the physicochemical properties of the fluid or the chosen programming technique. Of critical importance is the compatibility of the mobile phase with the SFC instrumentation. High quality stainless steels, such as Nitronic 60, should

be chosen to assure compatibility with most common fluids. Fluids such as NH_3, and even CO_2 containing trace amounts of H_2O, can corrode SFC system components. Sorption of supercritical fluids into elastomeric components, such as O-rings, can create leaks in the SFC system as well as dissolution of the elastomer into the fluid. For CO_2, this can be minimized by using components containing nitrile copolymers, Teflon polymer, or poly(ether ether)ketone (PEEK).

Detector compatibility is also a factor to consider when choosing a mobile phase for SFC. Since the flame ionization detector is the most widely used detector in SFC, care must be taken to purify the fluid to reduce the level of trace organic components. The use of a ultrahigh purity fluid or adsorbent cleanup train in this case should limit the degree of base-line rise in SFC when using the flame ionization detector (FID). The use of the FID in SFC, of course, precludes the use of organic modifiers and forces the analyst to consider using ultraviolet (UV) detectors equipped with high pressure flow cells.

The generation of mixed mobile phases can be avoided by purchasing prepared mixtures of organic solvents dissolved in a variety of supercritical fluids [70]. Blending of the organic modifier with the supercritical fluid can also be accomplished with the use of a binary syringe or piston pump arrangement. A novel laboratory method for preparing binary mobile phases has been reported by Raynie and co-workers [71].

4 INSTRUMENTATION

The basic instrumental configuration required for any analytical supercritical fluid system is minimally defined by the pressure region. There must be a pump to initially pressurize the system and to maintain supercritical pressure under dynamic operating conditions; and, at the end of the pressure zone, there must be some type of pressure restrictor through which decompression occurs. The extraction chamber (described in Section 5) for SFE or the chromatographic column for SFC is controlled within the supercritical pressure region by the pump and the restrictor.

In addition to the pump and restrictor, SFC requires a method to inject samples into the supercritical pressure region after the pump and before the column and a method to detect sample components after supercritical separation. The following sections discuss pumps, injectors, restrictors, and detectors commonly used in SFC. Requirements for pumps and restrictors are similar for both extraction and chromatography.

4.1 Pumping Systems

Pneumatic amplifier [28, 72], syringe [73], and reciprocating piston pumps [24, 32, 74, 75] have been used successfully with supercritical fluids. Pneumatic amplifier pumps have the advantage of ultrahigh pressure operation, reciprocating piston pumps have the advantage of continuous flow operation, and syringe

pumps have the advantage of pulseless flow and rapid pressure ramp capabilities.

Figure 1.10 provides a schematic diagram of a typical syringe pump used in SFC. When the piston is withdrawn, mobile phase from the supply tank fills the cylinder. The cylinder head is cooled to keep the mobile phase in the liquid state. Liquids are preferable for pumping, since they are more dense and less compressible than supercritical fluids or gases. The pumping rate is controlled with a drive screw that is connected to the motor either directly or through a gear train assembly. Computer control of the drive screw offers several advantages for SFC: pulseless flow, pressure or density programming, microflow rate control, and rapid pressure ramp operation. Dual syringe pumps can be used for composition gradient elution, but difficulties in correcting for mismatched solvent compressibilities can affect composition reproducibility, cross-contamination, and accuracy of the gradient.

4.2 Injection

Although improvements in pump design and operation are still needed for miximum efficiency and flexibility, it is injection methodology that remains the weak link in SFC instrumentation. The ideal chromatographic injector permits the nondiscriminating introduction of large volumes of sample at the head of the separation column in a zone that is negligible in width compared with the dynamic zone broadening processes that occur during separation. Although an injection method incorporating these ideal injection objectives has not yet been

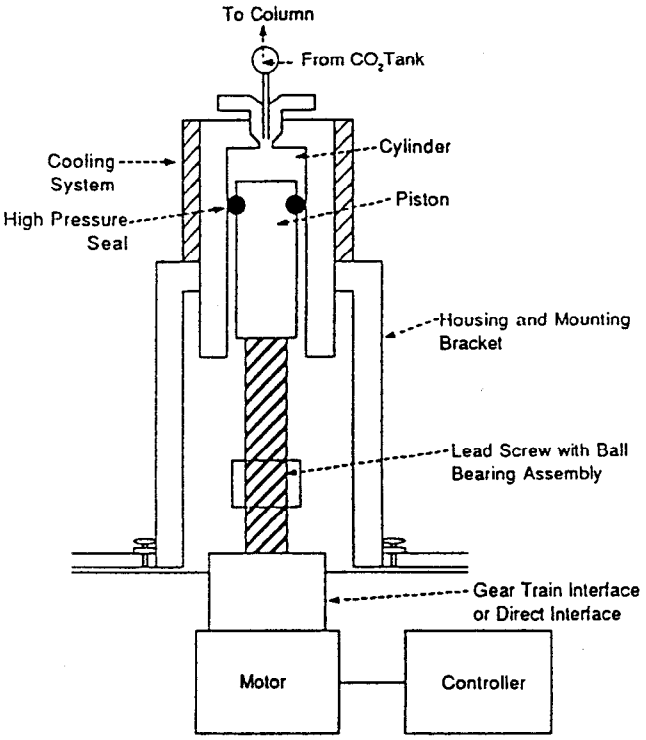

Figure 1.10 Schematic diagram of a syringe pump.

developed, a variety of injection methods do exist that offer individual injection advantages.

The most common injectors for SFC are high pressure valve injectors similar to those used in LC. With these valves the sample is loaded at ambient pressure into a sample loop of defined size. After the sample is loaded into the sample loop, the valve is switched to the inject position, placing the sample loop in line with the high pressure mobile phase flow, and the sample is swept from the loop into the column.

Direct injection of the sample from the loop into the column is the most desirable method. With this method the column is directly interfaced to the valve and quantitative transfer of the sample to the column is assured. Difficulties associated with direct injection methods include decreased separation efficiency from large volume injections, peak splitting, and detection interference from broad solvent peaks.

For open tubular columns (10-m × 50-μm i.d.) volumes less than 96 nL must be injected to limit loss in resolution to less than 1%. For packed columns, injection volumes of less than 1 μL should be used. For trace analysis larger injection volumes are often desired to achieve low detection quantities.

Peak splitting is generally caused by differences in solubilities of an analyte in the supercritical fluid and the sample solvent. When the analyte is more soluble in the solvent than in the supercritical fluid, it partitions between the supercritical fluid and the solvent. Some of it becomes adsorbed in the stationary phase at the head of the column and some of it moves ahead with the solvent plug until the solvent is completely evaporated and well mixed with the supercritical fluid.

Solvent interference due to large solvent injections can be a problem with the more quickly eluting compounds. The use of higher column temperatures and lower initial pressures help to focus the analytes at the head of the column while the volatile solvents pass through the column as a gas.

Quantitatively, solute focusing can be described by the following equation [76]

$$v = u\left(\frac{V_m C_m}{V_s C_s + V_m C_m}\right) = u\left[\frac{1}{1 + (K/\beta)}\right] \tag{11}$$

where v is the velocity of the solute band, u is the mobile phase velocity, V_s and V_m are the respective stationary and mobile phase volumes, C_s and C_m are the respective concentrations of the solute in the stationary and mobile phase, K is the partition coefficient described in (11), and β is called the phase ratio (V_m/V_s). Thus, if the partition coefficient of the solute decreases or the phase ratio of the column increases as the solute enters the column, its zone velocity will decrease and it will become focused at the head of the column. Temperature gradients [76–78], retention gaps [79, 80], and varying stationary phase thicknesses [81] have been used to focus solutes.

One approach for reducing peak splitting and focusing the solute is to place a mixing chamber between the injection valve and the column [82, 83]. This

provides time for the solvent to become diluted by the mobile phase, decreasing the solvent strength and increasing the partition coefficient. When the solute reaches the column, the phase ratio is decreased and the solute is focused at the head of the column.

While developments in direct injection continue, the most common method used for injection in SFC, especially with respect to open tubular columns, is split injection. Splitting the injection decreases the volume introduced onto the column and eliminates many of the problems associated with direct injection.

Several split injection methods are employed. Dynamic split is the simplest and most popular [80, 84–86]. This split assembly consists of a stainless steel tube connected directly to the injection valve. The other end of the stainless steel tube is connected to a tee. An open tubular column or transfer line is inserted concentrically through the tee and into the stainless steel tube. On the outlet of the tee is a restriction device, usually a fused silica restrictor, to control the flow split. The sample split occurs as part of the sample enters the open tubular column and part of the sample passes around the column and exits through the tee and split restrictor.

Advantages of the dynamic split are good resolution for complex mixtures and narrow solvent peaks. Disadvantages include nonlinearity, sample discrimination, and small volume injections. A timed-split method is commonly used to enhance linearity and decrease discrimination from split injections [80]. In timed-split injection, fast valve switching is used to permit only a fraction of the contents of the sample loop to be injected directly onto the column.

Various procedures for solvent elimination have been used in attempts to inject large sample volumes into the column. In one method, the sample is injected onto a precolumn where the solutes are selectively retained while the solvent is vented from the instrument through a restrictor [77, 87]. This venting process can be enhanced by purging with a gas until the solvent is evaporated and the solutes are precipitated on the walls of the precolumn [76].

Complete elimination of the sample solvent can be achieved with the backflush technique [76, 88]. With this approach, the split restrictor of a dynamic split injector described above is closed until the entire sample has entered the column. Then, the split is opened simultaneously with a rapid negative pressure ramp. This depressurization at the injector causes a reversal in flow at the head of the column and sweeps the solvent out of the split restrictor. All solvent elimination methods suffer from the this weakness. Volatile components can be partially eliminated with the solvent. Fortunately, most components of interest in SFC have relatively low volatility.

4.3 Restriction

To maintain supercritical pressure conditions in a chromatographic column or an extraction chamber, flow restriction must be applied on the downstream side of the column or chamber. Most systems utilize some form of fixed restrictors constructed of fused silica tubing. Figure 1.11 [89] shows schematic cross-sectional designs of several common fixed restrictors.

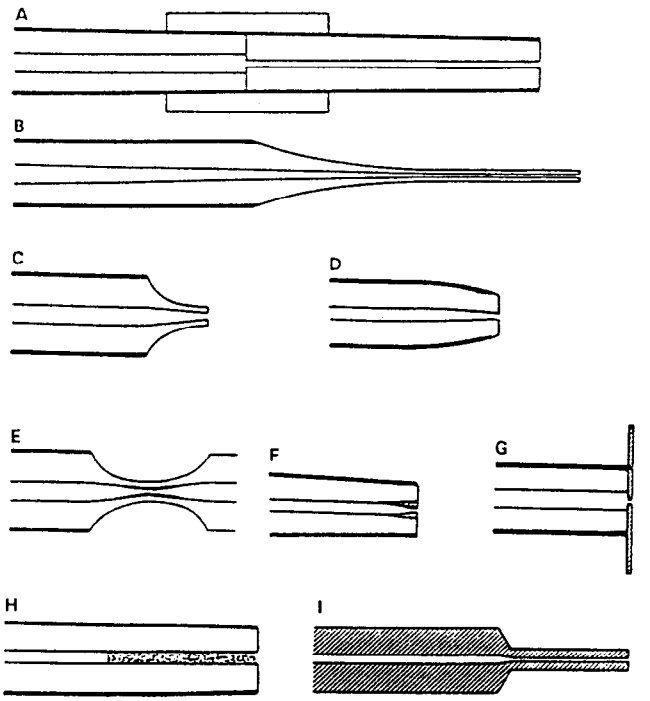

Figure 1.11 Schematic cross-section diagram of restrictor designs used in SFC and SFE: (*a*) Linear restrictor, (*b* and *c*) tapered fused silica restrictors, (*d*) integral restrictor, (*e*) converging-diverging restrictor, (*f*) deposition restrictor, (*g*) pinhole restrictor, (*h*) frit restrictor, (*i*) pinched restrictor. Reprinted with permission from B. W. Wright and R. D. Smith, in C. M. White, Ed., *Modern Supercritical Fluid Chromatography*, Huethig, Heidelberg, Germany, 1988, p. 189.

Figure 1.11(*a*) represents one of the earliest forms of flow restriction. Called a linear restrictor, it consisted of a fused silica tubing with a small internal diameter (e.g., 10 μm) that was butt connected to the column or transfer line [90]. The amount of restriction was determined by the ratio (aspect ratio) of the column length to its internal diameter. A primary disadvantage of linear restrictors is that decompression occurs throughout the length of the restrictor causing precipitation of some of the less volatile compounds.

Tapered restrictors, produced by drawing out the end of a fused silica column and shown in Figures 1.11(*b*) and (*c*), were developed to minimize the length of the decompression zone [79]. Integral restrictors [Figure 1.11(*d*)] decrease the decompression zone even further and are one of the two most preferred restrictor designs currently used with SFC [91]. The integral restrictor is constructed by polishing the closed end of a fused silica capillary until a small orifice is developed. The size of the orifice determines the flow characteristics of the restrictor. Other restrictors include the converging-diverging restrictor [Figure 1.11(*e*)] [92], the deposition restrictor [Figure 1.11(*f*)], the diaphragm restrictor with a laser drilled pinhole orifice [Figure 1.11(*g*)] [93], and the pinched restrictor [Figure 1.11(*i*)] [93]. The major problem with restrictors requiring extremely small orifices is that precipitated particles entrained in the fluid often cause plugging of the restrictor.

To minimize plugging and the decompression zone, the frit restrictor [Figure

1.11(*h*)] was developed [19]. Packed with an immobilized material, it produces multiple submicron fluid paths. Because of the multiple paths available to the fluid, this restrictor is not easily plugged by small particles.

All restrictors suffer from problems associated with the cooling that occurs from the expansion of the supercritical fluid as it decompresses. Restrictors must be heated to offset the effects of this cooling process. For SFE and compound identification applications, caution must be used when heating the restrictor. Too much heat may result in decomposition of the compound of interest.

In SFC the most common mode of operation is pressure or density programming to increase solvent strength during the chromatographic run. Unfortunately, as the pressure increases, the optimal chromatographic flow decreases. With fixed restrictors, the flow increases with pressure. Thus, at higher pressures chromatographic efficiency decreases. One approach to solve this problem is to increase restriction as the fluid pressure increases. The development of the ideal variable restrictor is not complete, but control of pressure on the downstream side of the restrictor is currently the most popular approach to vary restriction and supercritical fluid flow [24, 94–97].

4.4 Detection

Because supercritical fluids have liquid-like and gas-like properties, both LC detectors and GC detectors have been applicable to the detection of compounds after SFC. For a discussion of fundamental detection parameters important in SFC, see [98]. In general, LC detectors are adapted to SFC by using a closed cell design in which the fluid is maintained under pressure but cooled to a liquid before detection. Decompression occurs after detection. Gas chromatography detectors, on the other hand, have been adapted to SFC by using an open cell design in which the fluid is decompressed to a gas before detection. Whether GC-like or LC-like detectors are used for SFC detection, the solute should be introduced in a manner that permits maximum detector sensitivity and minimum band broadening of the peak as it passes through the transfer line, decompression zone, and detector cell. The SFC detectors can be divided into three categories: ambient pressure ionization, optical, and vacuum detectors.

Ambient pressure ionization detectors provide chromatographic response by monitoring changes in gas-phase ion currents as the solute passes through the detector. By far, the most useful SFC detector is the FID [99, 100]. When CO_2 is used as the mobile phase, the FID responds to most organic molecules with a detection limit for carbon of 5 pg/s and a linear dynamic range of 10^6. Other ambient pressure ionization detectors include the hydrogen–atmosphere FID for the detection of organometallic compounds [101], the thermionic detector for the detection of nitrogen and phosphorus containing organic compounds [90, 102–105], the electron capture detector for the detection of halogenated and other electronegative compounds [106, 107], the photoionization detector for the detection of organic compounds with ionization potentials less than 10.2 eV [108–110], and the ion mobility detector for molecular selective detection of compounds with high electron or high proton affinities [111–114].

The most common optical detector is the UV absorption detector (UVD) [115–118]. Although less sensitive than the FID and limited to compounds with chromophores, the UVD can provide compound-specific information and is nondestructive. Related to the UVD but much more selective and sensitive is the fluorescence detector (FD) [119]. A minimum detection limit of 500 fg/s has been reported for pyrene after SFC.

Fourier transform–infrared (FT–IR) spectroscopy is also possible after SFC in both on-line [120] and off-line [121] configurations. Carbon dioxide has extensive regions of transparency through which on-line detection can be made. However, CO_2 does have strong absorption bands from 3504 to 3822, 2137 to 2551, and below 800 cm^{-1}. In addition, Fermi resonance bands between 1225 and 1475 cm^{-1} increase in intensity at high densities. For on-line detection in these obscured regions, Xe has been used as the mobile phase [122], but solvent elimination with off-line detection provides a less expensive and more sensitive solution to the problem [121].

Gas-phase optical detectors include the flame photometric detector (FPD) for the detection of sulfur and phosphorus containing organic compounds [123, 124], the chemiluminescence detector (CD) for the detection of sulfur containing and easily oxidizable compounds [125, 126], and element selective plasma emission detectors [127–134]. The light scattering detector (LSD) has shown promise as a universal detector based on scattered light from nonvolatile compounds [135–137].

Finally, SFC instruments can be interfaced to vacuum detectors. The primary objectives for a vacuum interface to SFC are the following: (1) the large gas flow rates generated from the decompression of the mobile phase should be rapidly pumped away so that it does not unduly influence the detection process, (2) the solute must be efficiently transferred from supercritical pressure to vacuum conditions, and (3) the integrity of the chromatographic resolution must be maintained.

When a supersonic jet of mobile phase is expanded through an orifice into a low pressure region, a beam of cold molecules is produced. Laser induced fluorescence of these molecules produce information rich free-molecule spectra. This technique is known as supersonic jet spectroscopy (SJS) [138, 139].

The best known vacuum detection method after chromatography, however, is mass spectrometry (MS) [140–143]. A variety of ionization sources have been used with SFC/MS. The most common is chemical ionization (CI), but electron impact (EI), charge exchange (CE), atmospheric pressure ionization (API), thermospray and ion evaporation have also been investigated.

5 EXTRACTION

Analytical supercritical fluid extraction (SFE) involves the use of compressed gases, held above their critical temperature (T_c), for the extraction of analytes from a variety of sample matrices. The technique offers some unique advantages over conventional sample preparation techniques, particularly when CO_2 is

used as the extraction fluid. As noted in previous sections, the same properties that make supercritical fluids unique mobile phases for SFC, are also responsible for their performance when they are used in the extraction mode. For example, adjustment of the fluid pressure permits, to a degree, the selective extraction of specific analytes for subsequent analysis. Improvements in the kinetics of extraction are also realized by using supercritical fluids, due to the higher diffusion coefficients exhibited by solutes in the dense fluid media compared to their diffusivities in liquid–liquid extraction solvents. Recently, supercritical fluids have been cited as excellent extraction solvents, since their use avoids the problem of solvent waste disposal as well as exposure of laboratory personnel to toxic solvents [144].

Chronologically, analytical SFE developed somewhat later than SFC, although Stahl [145] reported on the coupling of SFE with thin-layer chromatography (TLC) as early as 1976. Supercritical fluid extraction has also been utilized by chemical engineers since the 1970s and the literature in this field [146–148] contains valuable information for the analytical chemist. Today, analytical SFE is practiced ranging from the submilligram to the 100-g level. Analytical SFE can be performed as an independent sample preparation technique or be coupled "on-line" to such chromatographic methods as GC and SFC. In this section we discuss the fundamental concepts governing this technique, its practice, and a sampling of the applications in which it has been used.

5.1 Fundamentals

Since analytical SFE is often used as an alternative for liquid–liquid extraction, it is useful to compare some of the physicochemical properties of supercritical fluids with those exhibited by typical liquid extraction solvents. In Table 1.9, some specific properties of CO_2 compressed to 200 atm at 55°C are compared to the condensed state values for commonly used liquid solvents. Note that the density of the compressed CO_2 is typical of values exhibited by liquid solvent

Table 1.9 Comparison of Physical Properties of Supercritical CO_2 with Liquid Solvents at 25°C

	CO_2[a]	*n-Hexane*	*Methylene Chloride*	*Methanol*
Density (g/mL)	0.746	0.660	1.326	0.791
Kinematic viscosity ($m^2/s \times 10^7$)	1.00	4.45	3.09	6.91
Diffusivity of benzoic acid ($m^2/s \times 10^9$)	6.0	4.0	2.9	1.8

[a]At 200 atm and 55°C.

media, but other parameters, such as the fluid viscosity and the diffusion coefficient of a typical solute, vary quite widely depending on the chosen solvent. It is the ability of supercritical fluids, such as CO_2, to achieve "liquid-like" densities coupled with their "more favorable" transport properties compared to liquid solvents that permit them to achieve higher extraction fluxes than those obtained using liquid solvents.

As noted in Section 3, the solubility characteristics of a supercritical fluid are directly related to its density. This relationship was used by Giddings and co-workers [35] to establish a "solubility parameter" scale, as shown in Figure 1.12, ranking highly dense gases in relation to the cohesive energy sensities of liquid solvents. Note that CO_2 at high densities can have similar solvent properties to such liquids as chloroform and acetone. At intermediate levels of compression, CO_2 and other supercritical fluids can emulate the solvent power of nonpolar hydrocarbons, such as *n*-pentane or diethyl ether. Such a scale explains the

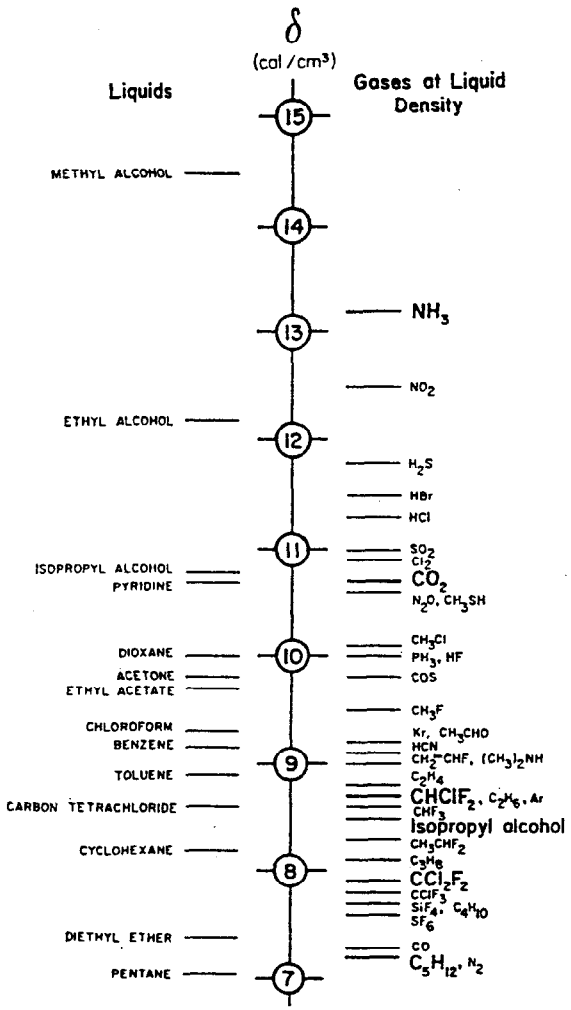

Figure 1.12 Solubility parameter scale of gases at liquid density compared to common liquid solvents. Reprinted with permission from J. C. Giddings, M. N. Myers, L. McLaren, and R. A. Keller, *Science*, **162**, 67 (1968). Copyright © 1968 by the AAAS.

qualitative features of supercritical fluids, but often leaves the impression that it is necessary to match the solubility parameters of the extraction fluid and solute to achieve a successful extraction [149]. Other methods have been developed to assist the analyst in choosing the density or pressure required for dissolving the solute in the supercritical fluid [150], or to predict the solubility of a solute in the fluid at a specific pressure or temperature [38, 151]. These concepts and the rules formulated by Stahl and co-workers [152] provide guidance in predicting the miscibility and extent of solubilization of a solute in a supercritical fluid.

A generalized solubility isotherm for a solute–supercritical fluid system as a function of pressure and at two different temperatures, T_1 and T_2, is shown in Figure 1.13. Upon initial pressurization of the system, there is a decrease in solute solubility in going from the respective pressures designated by points A and A' to B and B'. At a certain pressure beyond B and B', the solute's solubility begins to increase with pressure. Frequently, this pressure regime is called the "threshold pressure" [28], since there is a large measurable solubility enhancement of the solute in the dense fluid solvent. However, it has been noted [153] that the above-reported solubility trends and threshold pressures are very dependent on the technique that is utilized to measure the solute's solubility in the supercritical fluid media. However, the differential extraction behavior

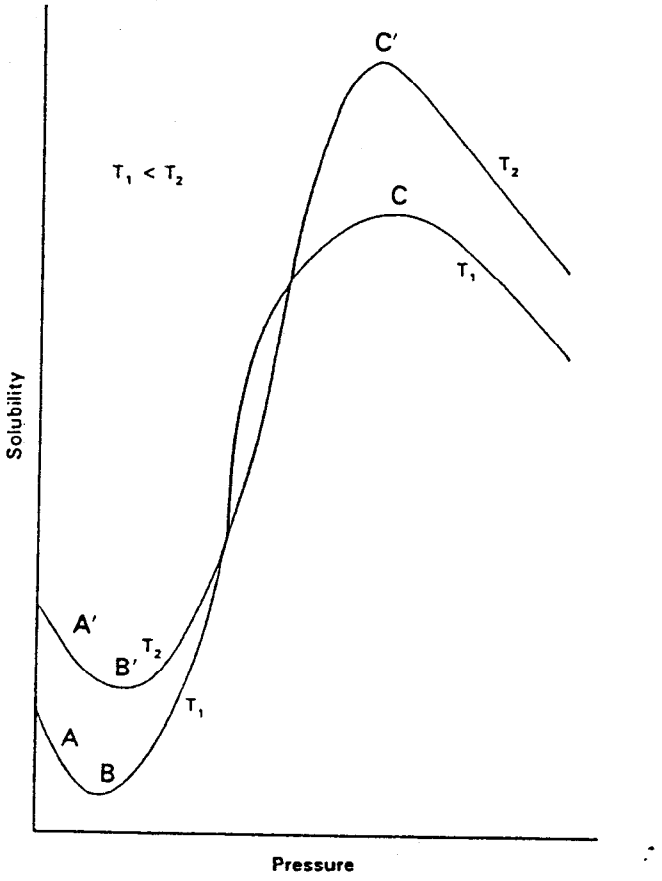

Figure 1.13 Generalized solublity isotherms as a function of pressure.

exhibited between points A and A' or B and B' can obviously be used as a basis for the selective extraction of target analytes.

Similarly, fractionation of solute mixtures can be performed in the pressure interval between B or B' and C and C', although the relative separation factor between individual solutes α is not always large. Note that the solubility isotherms may cross at a particular pressure called the "cross-over pressure" [154], at which the solubility of one solute can be enhanced in the fluid phase relative to the other. Solute fractionation at the solubility maxima, C and C', as shown in Figure 1.13, is also possible, but the resultant α values may be low, since many solutes will extract into the supercritical fluid at these high pressures. For this reason, some analysts avoid conducting extractions in the solubility maxima region. However, as shown by King and co-workers [155], this pressure region is to be preferred for exhaustively extracting bulk phases, such as lipid materials from insoluble sample matrix components. Also, extractions conducted in this region generally can be completed much more rapidly, since the solutes have considerably higher solubilities in the supercritical fluid under these conditions.

Of equal importance in the above solubility criteria are the mass transfer properties of the extracted solutes in the supercritical fluids. Solute extraction fluxes from a sample matrix are directly proportional to the product of the solute's solubility in the supercritical fluid times its diffusivity in the fluid. Therefore, as a solute's solubility increases with pressure, its corresponding diffusivity in the supercritical fluid can decrease over two orders of magnitude. The net effect of the above two trends can best be measured in terms of mass transfer coefficients or dimensionless transport numbers. For example, the ratio of the Reynolds number (Re) for CO_2 at 200 atm and 55°C to those for the liquid solvents cited in Table 1.9, at an equivalent fluid velocity, is 6.5, 5.0, and 1.74 for methanol, *n*-hexane and methylene chloride, respectively. In this case, the larger fluid turbulence that occurs in the CO_2 should greatly enhance the rate of solute extraction.

The kinetics for solute extraction into a supercritical fluid follow a similar pattern to that observed for liquid extraction. As shown in Figure 1.14, the initial stage of the extraction is governed by the distribution coefficient of the solute between the dense fluid phase and the sample matrix, giving way to a diffusion-controlled process in the latter stages of the extraction. The implications of the curve shown in Figure 1.14 on the extent and time of SFE has been treated theoretically by Bartle and co-workers [156] in terms of the "hot ball" model, where the mass of extractable material remaining in the sample matrix m to the mass of original extractable material m_0, is given by

$$\frac{m}{m_0} = \left(\frac{6}{\pi^2}\right) \sum_{n=1}^{\infty} \left(\frac{1}{n^2}\right) \exp\left(\frac{-n^2\pi^2 Dt}{r^2}\right) \qquad (12)$$

where n is an integer; D is the diffusion coefficient of the solute in the hypothetical spherical matrix of radius r; and t is the extraction time. This

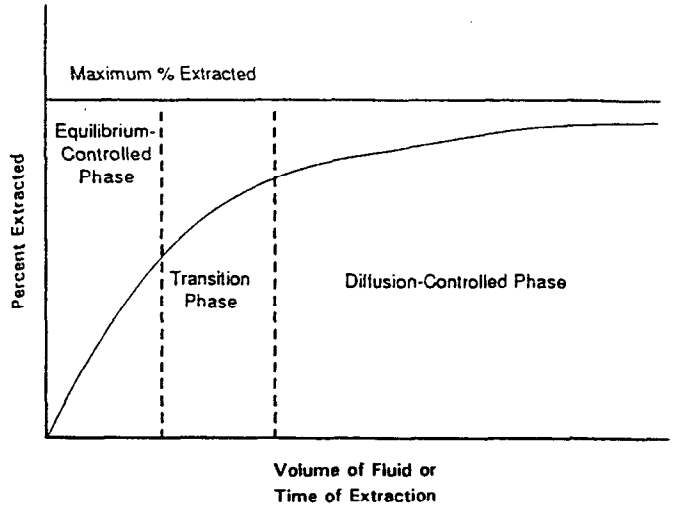

Figure 1.14 Generalized extraction curve of percent solute extracted as a function of volume of extraction fluid or time of extraction.

expression can be rewritten in terms of reduced time $t_r = \pi^2 Dt/r^2$, to yield an expression for m/m_0 in terms of an exponential decay series expansion. The final expression, given in (13) is

$$\frac{m}{m_0} = 6\pi^2[\exp(-t_r) + \exp(-4t_r) + \exp(-9t_r) + \cdots] \tag{13}$$

The latter equation has been found to describe analytical SFE kinetics from such diverse sample matrices as railroad bed soil, crushed rosemary, and comminuted polypropylene pellets.

In many cases, slow solute extraction kinetics or limiting analyte solubility in the fluid phase, can be overcome by the addition of modifiers or cosolvents to the supercritical fluid phase. Examples of solubility enhancements for selected solutes that have been realized by adding modifers into supercritical CO_2 are shown in Table 1.10. The addition of methanol to CO_2 not only enhances the solubilization of polar solutes, such as acridine and 2-amino benzoic acid, but increases the solubility of highly soluble lipophilic solutes, like cholesterol, over 100-fold. Certain specific modifiers, such as tributyl phosphate, act as complexing agents [157], thereby enhancing the extraction of a donor molecule, hydroquinone, over 300-fold.

5.2 The Practice of Supercritical Fluid Extraction

Supercritical fluid extraction has been implemented in the laboratory using a variety of methods and equipment. However, a generic SFE apparatus, as shown in Figure 1.15, usually consists of a pump or compressor, an extraction vessel, a pressure reduction device, followed by a collection vessel. Analytical SFE is usually practiced in either an off-line or on-line mode, the latter

Table 1.10 Solubility Enhancements in Supercritical Carbon Dioxide with Various Modifiers[a]

Solute	*Modifier*	*Enhancement Factor*
Acridine	3.5% MeOH	2.3
2-Amino benzoic acid	3.5% MeOH	7.2
Cholesterol	9% MeOH	100
Hydroquinone	2% Tributyl phosphate	> 300
Tryptophan	0.53% AOT, $W_0 = 10$ 5% Octanol	≫ 100

[a]Reprinted with permission from K. P. Johnson, M. L. Penninger, Eds., *Supercritical Fluid Science and Technology* (1989). ALS Symposium Series 400. Copyright © 1989 American Chemical Society.

technique usually consisting of the direct transfer of extract into a chromatographic column. The choice between the two SFE techniques will be dictated by the problem facing the analyst; however, there is some merit in starting with the off-line mode because of its inherent simplicity. In general, off-line SFE permits the processing of larger sample sizes, while sample size in on-line SFE is dictated by the constraints of sample overload on the chromatographic column. On-line SFE is more prone to contamination, but is inherently more sensitive than off-line techniques. This makes on-line SFE coupled with SFC or GC an excellent technique for characterizing extremely small samples [158].

Figure 1.16 illustrates a typical off-line SFE apparatus with provision for collecting the extracted analytes in a vial after decompression of the extraction fluid. In this scheme, the extraction fluid is delivered through a pump, whose heads are cooled to a subambient temperature to assure liquefaction of the gas. A second pumping module is also connected in-line to provide for the addition of a modifier (methanol in this case) to the extraction fluid. The extraction vessel is contained in a heater assembly that equilibrates the extraction fluid to the desired temperature. After extraction, the fluid pressure is jettisoned through a back pressure regulator, and the analytes of interest are collected in a solvent-laden vial, cooled by the adiabatic, isoenthalpic expansion of the expanded fluid

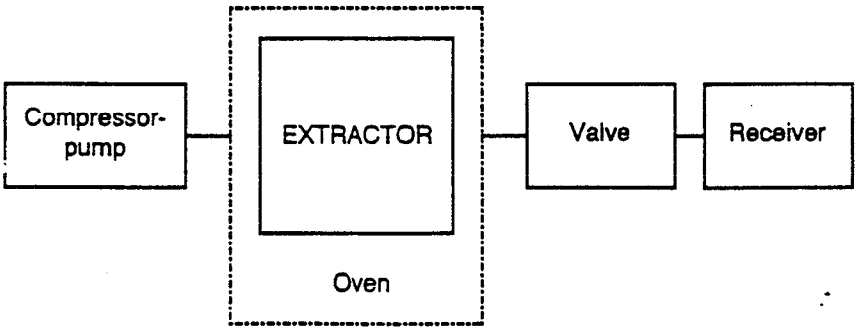

Figure 1.15 Generalized supercritical fluid extraction system.

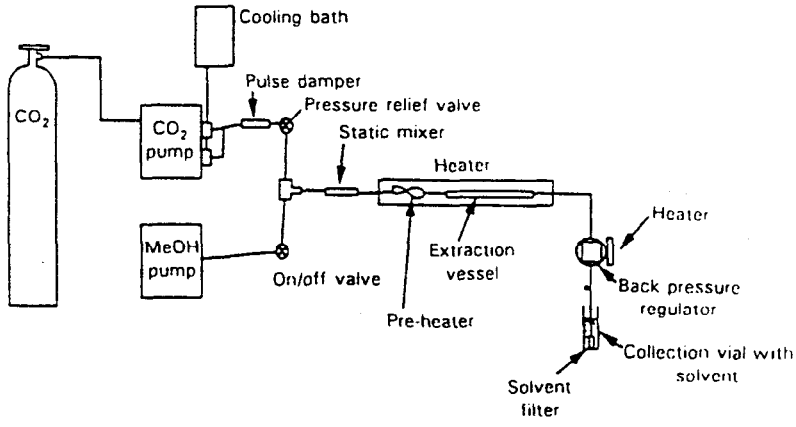

Figure 1.16 Diagram of a typical off-line supercritical fluid extraction system.

stream. The described off-line SFE apparatus has been utilized recently to extract lipids from human skin [159].

The on-line mode of SFE is illustrated in Figure 1.17 where a single pump is used to deliver the fluid for both the extraction and the chromatographic steps. For the apparuatus in Figure 1.17, the multiport switching valve in conjunction with the column shut-off valve permits the diversion of the fluid either into the extraction cell or the column proper. Extract from the cell is subsequently trapped in a tee before commencement of the chromatography step. This is accomplished by reswitching the valve so as to effect the desorption of the sample from the CO_2 cooled tee onto the head of the column. In this particular

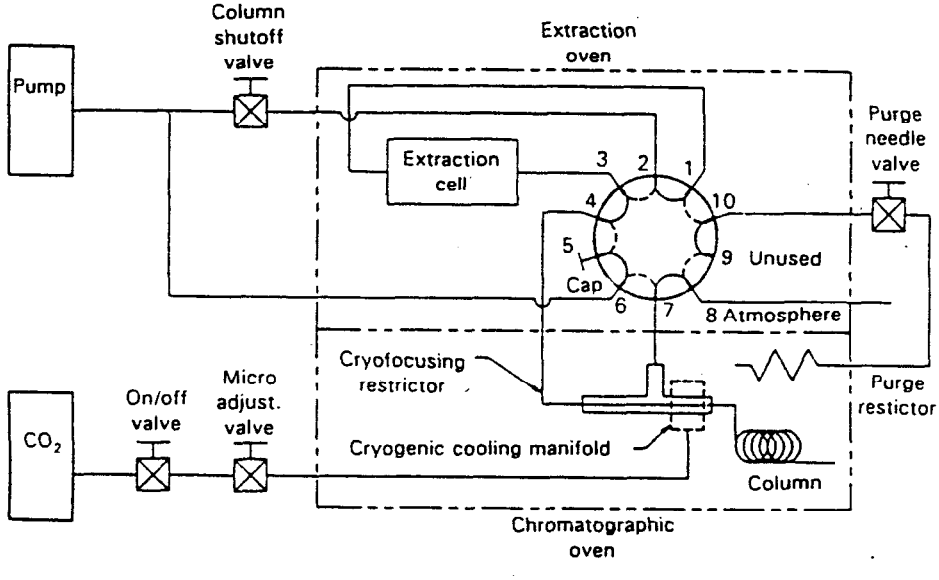

Figure 1.17 Schematic representation of a typical on-line supercritical fluid extraction apparatus. Reproduced from the *Journal of Chromagraphic Science* by permission of Preston Publishers, A Division of Preston Industries, Inc., Niles, IL.

configuration, the cell may be purged with extraction fluid while the chromatographic analysis is being performed [160].

Many variations of the above on-line and off-line equipment exist. For example, King and co-workers [155] described an off-line system utilizing a gas booster pump that can extract 250-gram samples at pressures up to 700 atm. The lipid extracts obtained from heterogeneous food samples on such a system are then assayed for pesticide residues. "Pumpless" off-line systems [161–163] have been employed for the low pressure SFE of trace levels of pesticides in meat and fish samples, using either CO_2 at normal tank pressures or heated, comminuted dry ice as sources of the extraction fluid. On-line SFE systems can be quite complex, involving integrated cleanup steps or multidimensional column approaches to separate complex extracts. Toward this end, Nam and Kapila [164] coupled SFE with SFC followed by GC for the separation of fractions obtained from SFC. This system has been applied to the analysis of organochlorine pesticides and polychlorinated biphenyls present in fish tissue. A similar approach has been utilized by Murugaverl and Voorhees [165] in which a packed column is used to fractionate the SFE extract before analysis by SFC. A tandem combination of SFE with SFC and MS has allowed Ramsey and co-workers [166] to analyze for drug residues in kidney tissue. Similarly, SFE has been used on-line with microcolumn LC [167] for the analysis of polymer additives.

Several factors must be considered and optimized to conduct a successful SFE. The effect of such variables as pressure, temperature, and the choice of supercritical fluid or modifier have been noted previously. The flow rate at which the extraction is conducted should be commensurate with the sample size and the kinetics of the extraction. Most analytical SFE is conducted in the dynamic mode with supercritical fluid flowing continuously through the extraction cell. However, for severe rate limited extractions, the static mode of extraction may be preferred. Wheeler and McNally [168] reported excellent recoveries of pesticides from soil samples using static SFE.

Two factors that can have a considerable impact on the results obtained with SFE are the type of collection technique that is employed and the influence of the sample matrix on the efficiency of the extraction. Table 1.11 categorizes the analyte collection techniques that have been used to date into four generic classes. The most popular collection method involves total depressurization of the extract into a suitable collection device. For off-line SFE, this frequently involves collection in an open reservoir to encourage nucleation of the extract, or dissolution of the expanded gas stream in a liquid solvent whose evaporation is minimized by the Joule–Thomson cooling attending the expansion of the extraction fluid. Adsorbent columns or cryocooling are both utilized in off- and on-line SFE; however, both techniques can discriminate on the basis of "light–heavy" molecular selectivity [169], which introduces a bias into the final extract that is obtained. On-column collection is favored in on-line SFE, where the extract is concentrated on a retention gap [76].

Table 1.11 Analyte Collection Techniques

Total depressurization
Solvent
Solid adsorbent column
Cryofocus trap
Open reservoir
On-column (capillary GC)
Partial depressurization (cyclical SFE)
Reservoir
Sample loop
No depressurization
Ion exchange resin
Temperature adjustment
Reservoir

Although not widely practiced to date in analytical SFE, partial depressurization of the extract into a reservoir or sample loop, with subsequent recycling of the extraction fluid back to the extraction cell is feasible. This collection method should find use in processing larger samples and/or as a method to concentrate low levels of target analytes. Recirculation of the extraction fluid over a sample to establish an equilibrium concentration of the extract in the circulating fluid has been reported by Engelhart and Gargus [170]. In this case, an aliquot from the extraction loop is taken through a sampling valve for either off- or on-line analysis. A similar approach has been described by Sugiyama and co-workers [171] for a directly coupled SFE–SFC system. Pressurized fraction collection that does not involve fluid recycle has been reported by Campbell and Lee [172] for the isolation of crude oil fractions.

Analyte isolation can also be accomplished at the same pressure used for SFE. Trapping of the analytes in this case is facilitated by the inclusion of a sorbent-filled cartridge between the extractor vessel and the pressure reduction valve. The chosen sorbent must have a strong affinity for the target analytes so as to effect a quantitative partition of the analyte onto the sorbent in the presence of the high pressure fluid. Schaeffer and co-workers [173] used the above principle for isolating alkaloids from the total fluid extract derived from plants. One of the advantages of this collection method is that undesired coextractives, such as lipid moieties, can be removed by SFE, while the target analytes are isolated on an ion exchange resin for subsequent elution and analysis.

Several sample matrix parameters influence the end result that is obtained by SFE. These parameters are listed in Table 1.12. As would be expected, sample particle size is crucial in determining the efficiency of the resultant SFE, as predicted by (12). In general, a finely comminuted sample promotes rapid SFE, due to the improved mass transfer of the solute into the flowing fluid stream. The

shape of the sample particle also influences the kinetics of extraction. In some uses of SFE, a spherical particulate shape is not the preferred configuration for rapid and complete SFE [174]. Samples having large surface areas generally extract easier than substrates devoid of surface area or porosity. These latter two parameters are subject to perturbation during the course of the extraction, since fluids held at high pressures can penetrate the sample matrix and change its original morphology [175].

The presence of water in the sample matrix can alter the results that are obtained with SFE. Literature results suggest that the presence of moisture in some samples can accelerate the removal of polar analytes from a variety of natural products [176]. King [177], using the on-line SFE–SFC option, has shown that widely different chromatographic profiles are obtained for extraction of organic matter from soil matrices depending on the water level in the aquifer sample. Moisture actually inhibits the extraction of lipophilic moieties from food products [155], since the extraction fluid encounters difficulty in contacting the lipophilic phase through the surrounding water layer.

Sample size and the level of extractable material are also factors that exert an influence in both off- and on-line SFE. To date, on-line SFE has been mostly conducted using syringe pumps, small extraction cells, and microbore column technology. Such equipment has predicated the use of small sample sizes, due to the limited fluid delivery capacities of syringe pumps and the sensitivity of microbore columns to sample overload. For this reason, the SFE of nonhomogeneous samples should best be undertaken with the off-line mode of SFE, which more readily accommodates a variable sample size. Similar considerations also apply for the level of extractables in a sample. Since solute solubilities in supercritical fluids can range over many orders of magnitude, it is difficult to scale an analytical SFE properly unless one has prior knowledge of the molecular identity and amount of extractable material. For this reason, sample sizes are frequently kept small for on-line SFE to avoid overloading a chromatographic column.

Table 1.12 Sample Matrix Parameters that influence Supercritical Fluid Extraction

Particle size and shape
Surface area and porosity
Moisture content
Changes in morphology
Sample size
Extractables level

5.3 Applications of Supercritical Fluid Extraction

Analytical SFE has produced a plethora of applications over the short time that it has existed. Representative applications abound in such diverse areas as polymer characterization, food analysis, flavor and fragrance chemistry, and the environmental sciences. Several useful references [159, 178, 179] are available that cite numerous applications of both on- and off-line SFE. For this reason, this section avoids citing numerous applications of SFE and focuses instead on selected applications that illustrate concurrently the technique and breath of SFE.

It was noted earlier that CO_2 could be compressed to densities that yielded equivalent solvent strengths to those exhibited by liquid solvents, such as *n*-hexane and methylene chloride. Figure 1.18 shows a GC comparison of a cardamon oil extract obtained from an *n*-hexane extraction versus an off-line CO_2 extraction [180]. The resultant chromatograms are remarkably similar, verifying the equivalent solvent power of CO_2 to *n*-hexane. However, the CO_2 extract GC profile contains some additional flavor notes, particularly at the beginning of the programmed temperature GC run, which are absent in the liquid-derived extract. This result is not unexpected, since SFE has been shown to yield natural product extracts, free of processing artifacts [181].

Table 1.13 gives the results of performing an off-line SFE of peanut butter containing incurred pesticide residues [182]. In this particular case, the peanut butter oil was extracted at 680 atm and 80°C, conditions that are optimal for

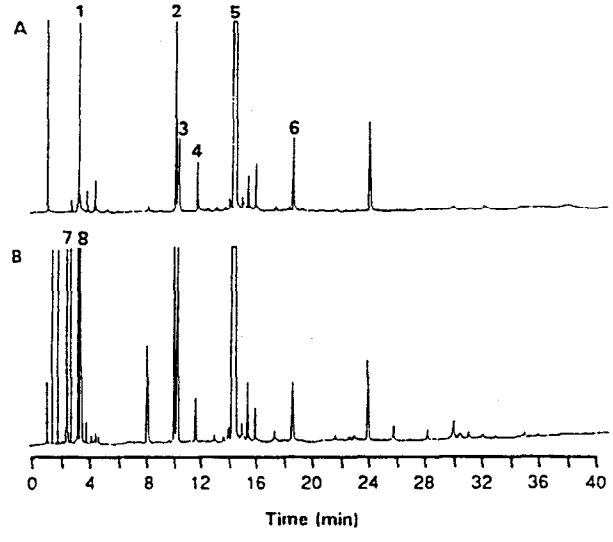

Figure 1.18 Gas chromatograms of cardamom oil extracted with (*A*) liquid hexane and (*B*) supercritical CO_2. Gas chromatographic conditions: open tubular column, poly(ethylene) glycol stationary phase; temperature program from 80 to 140°C/min at 4°C/min and to 280°C at 10°C/min after a 5-min isothermal period. Peak identifications: (1) limonen, (2) linalool, (3) linalylacetate, (4) terpinen-4-ol, (5) terpinylacetate, (6) geraniol, (7) sabinen, and (8) 1.8-cineol. Reprinted with permission from K-W. Quirin and D. Gerard, in *Grenzen, Moglichkeiten und Anwendungen uberkritischer Phasen in der Chromatographie*, Saarbrucken, Germany, 1988.

Table 1.13 Analysis of Incurred Pesticides in Peanut Butter[a] with Supercritical CO_2 Extracted from Extraction Enhancer[b]

Pesticide	*Original Analysis*[c] (ppm)	*Supercritical Fluid Extraction* (ppm)
Penta Cl benzene	0.0008	0.0010
HCB	0.0004	0.0004
Penta Cl anisole	0.0007	0.0001
Quintozene	0.0004	
Penta Cl aniline	0.0020	0.0021
Penta Cl thio anisole	0.0008	0.0002
Heptachlor epoxide	0.0006	0.0004
Dieldrin	0.0030	0.0032
Chlorpyrifos	0.0070	0.0049
p,p-DDE	0.0020	0.0021
Toxaphene	0.1200	0.0800

[a]Single determination.
[b]Data taken from [182].
[b]Conventional organic solvent extraction.

solubilizing triglyceride-based oils in supercritical CO_2 [183]. An inert diatomaceous dispersant, called an extraction enhancer, was added to the sample before it was placed in the extraction cell to facilitate rapid extraction of the pesticide-laden oil. The lipid phase was extracted in 15 min, fractionated by column chromatography, and the pesticide fraction was analyzed by GC using an electron capture detector. A comparison of the SFE results with those obtained by conventional organic solvent extraction indicates excellent agreement for these parts per billion level recoveries.

An example of an on-line SFE/SFC application is illustrated in Figure 1.19 for the analysis of an aqueous solution of prostaglandins [184]. The aqueous sample in this case, containing approximately 13 ppm of prosequendum, was placed on a dispersant, a C_{18} packing material, and extracted with CO_2 at a density of 0.80 g/mL and 45°C for 45 min. The resultant supercritical fluid chromatograms show that the isopropyl ester of prosequendum was quantitatively extracted during the first extraction, but the parent compound, prosequendum $F_{2\alpha}$, was not desorbed even after performing a second extraction. These results show that SFE of selected analytes from adsorbents can be difficult; however, it also indicates that sorbents can be used to fractionate mixtures during SFE.

Further evidence concerning the complexity of extracting analytes from active sorbent surfaces is shown in Figure 1.20 for on-line SFE/GC analysis. Here a test mixture of aliphatic and aromatic hydrocarbons was spiked on an alumina sorbent and separate extractions were performed using CO_2 and SF_6

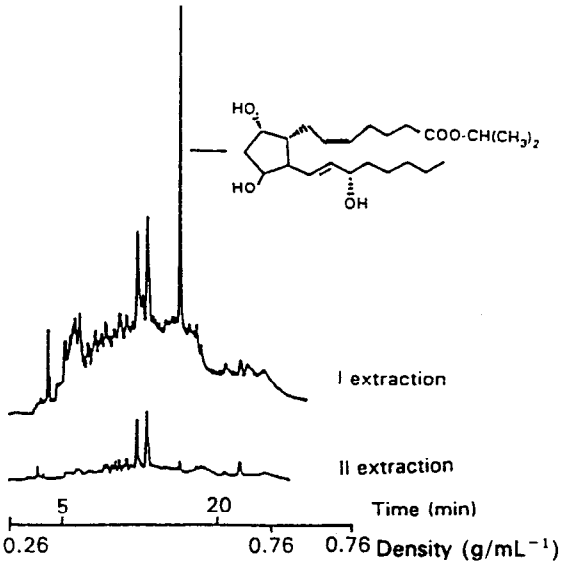

Figure 1.19 Supercritical fluid extraction of prostaglandin $F_{2\alpha}$–isopropyl ester and prostaglandin $F_{2\alpha}$.

[185]. The extraction conditions, which were actually subcritical (375 atm and 25°C), yielded vastly different results for each of the supercritical fluids. In the case of the SF_6 extraction, only a portion of the original spike, the *n*-alkanes were removed by SFE. However, the CO_2 extraction readily extracted both types of solutes from the alumina surface. One possible explanation for these differing results is the closer proximity of the extraction temperature to the critical temperature of CO_2. This results in an increased extraction flux as the T_c of the fluid is approached.

The results recorded in Figures 1.19 and 1.20 partially illustrate the difference between solute solubility and extractability in SFE. Solubilizing a solute in a supercritical fluid does not guarantee that it will be extracted successfully from a specific sample matrix under the same conditions. Taguchi and co-workers [186] showed that *n*-alkyl phthalates partition into dense CO_2 quite differently depending on the sample matrix from which they are extracted. For example, good recoveries are recorded when CO_2 is used for the extraction of pthalates from glass wool or filter paper. However, extraction of these solutes under the same conditions from activated carbon was impossible. Even increasing the extraction pressure, temperature, or time (up to 2 h) did not result in successful recovery of spiked analytes from the activated carbon. Direct addition of small quantities of carbon disulfide to the extraction cell, however, did increase the extractability of the phthalates.

Another variant of on-line SFE is shown in Figure 1.21, where an extraction has been performed on a single live beetle. The sample in this case was subjected to cooling to lower the metabolic rate of the insects before their insertion into the extraction cell [158]. Extractions using CO_2 were performed at 200 atm and 45°C for 1 min, the resulting extract being then cryofocused at the head of an

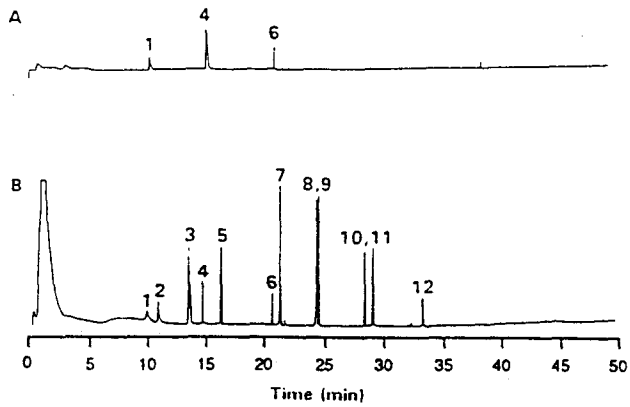

Figure 1.20 The SFE/GC chromatograms of aliphatic and aromatic hydrocarbon test mixture obtained by (*A*) SF_6 extraction and (*B*) CO_2 extraction; SFE conditions: sample adsorbed on alumina, 375 atm, 25°C and 30 min; GC conditions: 50-m × 200-μm i.d. open tubular column, polymethylsiloxane stationary phase (d_f = 0.5-μm); 30°C for 8 min, then to 310°C at 7°C/min; FID. Peak identifications: (1) *n*-decane, (2) butylbenzene, (3) naphthalene, (4) *n*-dodecane, (5) 1-methyl naphthalene, (6) *n*-pentadecane, (7) fluorene, (8) phenanthrene, (9) anthracene, (10) fluoranthene, (11) pyrene, and (12) chrysene. Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, a division of Preston Industries, Inc., Niles, IL.

SFC capillary column. A pressure programmed SFC run yielded the profile shown in Figure 1.21. The early eluting skewed peak is a communication pheromone emitted by the insect to encourage aggregation, while the late eluting peaks are some of the cuticular wax components that comprise the insect's shell. This cited case is an excellent example of the application of on-line SFE/SFC to small samples.

An interesting comparison of SFE results obtained on a common sample, a standard reference material consisting of polynuclear aromatic hydrocarbons

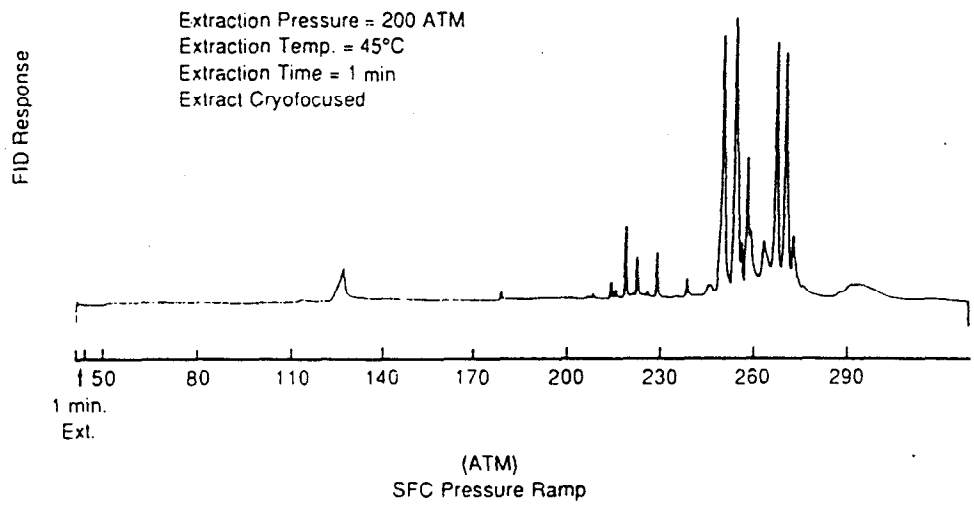

Figure 1.21 An SFE/SFC separation of the on-line CO_2 extract from a single dried fruit beetle (live specimen). Reproduced from the *Journal of Chromagraphic Science* by permission of Preston Publications, a division of Preston Industries, Inc., Niles, IL.

Table 1.14 Quantitation of PAHs from Urban Air Particulates (NIST SRM 1649)

	PAH concentration (μg/g)			
PAH	*Certified Value*[a]	*Off-Line* SFE[b]	*Split* SFE/GC[c]	*On-Column* SFE/GC[d]
Fluoranthene	7.1±0.5	8.0±0.6	7.2±0.9	7.3±1.0
Benz[*a*]anthracene	2.6±0.3	2.9±0.5	2.6±0.8	2.6±0.8
Benzo[*a*]pyrene	2.9±0.5	3.2±0.3	2.7±0.4	2.8±0.5
Benzo[*ghi*]perylene	4.5±1.1	4.4±0.3	3.9±1.0	3.6±0.9
Indeno[1,2,3-*cd*]pyrene	3.3±0.5	3.1±0.2	3.4±0.6	3.0±0.5

[a]Value certified by the National Institute of Science and Technology (NIST) based on a 48-h Soxhlet extraction of a 1-g sample.
[b]Based on triplicate 60-min extractions of 20-mg samples at 350 atm with N_2O/5% MeOH. Values were adapted from [187].
[c]Based on 30-min extractions of 50-mg samples at 375 atm and 50°C with CO_2. Values were adapted from [185].
[d]Based on four replicate analyses of 2-mg samples using SFE/GC/MS with supercritical N_2O. Each extraction was performed at 350 atm and 45°C. Results are adapted from [188].

(PAHs) adsorbed on urban air particulates, is reported in Table 1.14 [185, 187, 188]. Here a cross comparison is made of the extraction results obtained from a conventional Soxhlet method, with those obtained from off-line SFE, split SFE/GC, and on-column SFE/GC. The results are in excellent agreement within the limits of the reported standard deviations. This lends further credence to the reproducibility of SFE techniques and the promise that they hold for analytical chemistry.

6 APPLICATIONS

6.1 Introduction

It would be impossible to cover in detail all applications of SFC and SFE in this chapter. Instead, selected examples of applications have been grouped into different categories to cover the main principles and techniques. It is hoped that the reader will gain insight primarily into the fundamental principles governing the practices of SFC and SFE, rather than the details concerning the analysis of specific samples.

6.2 Fossil Fuels and Hydrocarbons

Analytical SFC offers new opportunities for hydrocarbon analysis that arise from the availability of the FID, and from the possibility of achieving high resolution of heavy hydrocarbons that exceed the analysis range of GC. Hydrocarbon type analysis primarily refers to the separation and quantitative measurement of saturates, olefins, aromatics, and polar components. Polar

compounds include heavy condensed aromatic hydrocarbons and compounds containing sulfur, nitrogen, and oxygen heteroatoms. Before SFC was available, most methods for hydrocarbon type analysis were based on LC with packed columns operating in the adsorption mode. The main drawback of the LC techniques has been the lack of a suitable detector for easy and accurate quantitation. In combination with the FID, SFC has provided a solution to this problem, since the detector, widely used in GC, has been shown to have a nearly uniform response for hydrocarbons. No single column has been developed to date that separates aliphatics, olefinics, and aromatics completely.

Incorporation of a silver-loaded, strong cation exchange silica column, in addition to another silica column, and valves for column switching and backflushing, as well as the use of a mixed SF_6/CO_2 solvent, led to an analysis method that achieved adequate separation of all types in 50 min (Figure 1.22), and it was also applicable to middle distillates as well as more volatile fractions [189]. However, the instrumentation was somewhat complex, and SF_6 is an environmentally objectionable fluid.

A new SFC method for hydrocarbon type analysis has recently been developed that retains the simplicity of a single silica column operated with CO_2 as the mobile phase. This method [190] employs UV absorption and flame ionization detectors in series, where the UV detector acts as an olefin-selective detector and the FID acts as a general mass sensitive detector for all three hydrocarbon types. It has been shown that by operating the UV detector at

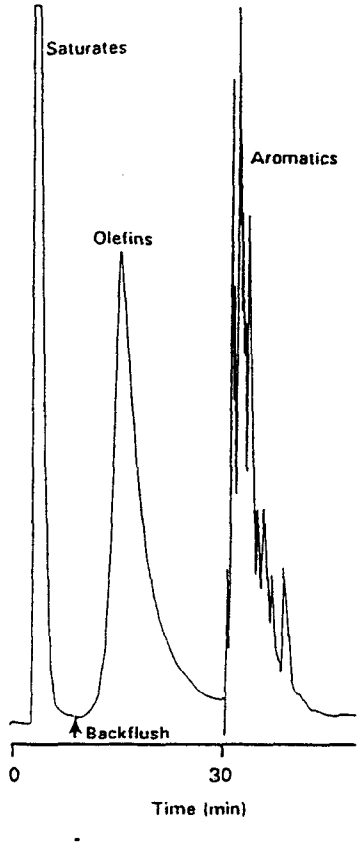

Figure 1.22 An SFC/SFC chromatogram of a gasoline sample showing hydrocarbon group-type separation. Conditions: 25-cm × 1-mm i.d. packed silica and silver-loaded sulfonic acid silica microbore columns; 10 mol% CO_2 in SF_6; FID. Reprinted with permission from R. M. Campbell, N. M. Djordjevic, K. E. Markides, and M. L. Lee, *Anal. Chem.*, **60**, 356 (1988). Copyright © 1988 American Chemical Society.

190 nm, olefins of the types present in gasoline can be measured not only with high sensitivity but with a uniform detector response that permits quantitation of the olefin fraction.

Standardized GC methods (i.e., simulated distillation) exist for the measurement of boiling point distributions of petroleum fractions boiling to 565°C at atmospheric pressure. The need to support heavy hydrocarbon conversion processes has been the driving force to extend simulated distillation to higher temperatures.

The promise of SFC in performing simulated distillations under mild, nondegradative conditions and in extending the analysis to even higher boiling materials generated considerable research activity. Both with open tubular and packed columns, separations of species up to and exceeding 100 carbon atoms were achieved under temperature conditions that did not exceed 150°C.

However, a number of obstacles were encountered that prevented broad application of the technique. Low temperatures in the injector caused precipitation of wax components and memory effects due to sample carryover. Some heavy fractions proved to be insoluble in CO_2, even at high densities.

Recent advances have helped solve some of these problems and have given SFC simulated distillation new impetus. Direct injection and other automated injection modes, such as timed-split, have eliminated or greatly reduced discrimination. The availability of heated injectors has largely eliminated the precipitation of high molecular mass components. Use of higher boiling solvents, such as *n*-decane, for initially dissolving heavy samples, has been helpful.

The analysis of polycyclic aromatic compounds (PACs), widely found in fossil fuels and combustion products, are of interest because of their alleged carcinogenic properties. Open tubular column SFC, with its inherent advantages of low pressure drop and high resolution has been applied to the separation of PACs with more than 10 aromatic rings [191].

Open tubular columns containing bonded liquid crystalline polysiloxane stationary phases have achieved PAC resolution superior to that obtained by GC with the same phases because of the more ordered structure of the stationary phase at the low temperatures used in SFC (Figure 1.23).

Juvancz and co-workers [192] reported an on-line, two-dimensional SFC system for a packed capillary column and an open tubular column separation of complex PAC mixtures. The packed capillary in the first dimension, being an aminosilane-bonded silica column, provided a rapid chemical class separation according to the number of aromatic rings, while the open tubular column in the second dimension, being a liquid crystalline polysiloxane column, provided high resolution of closely related isomers.

6.3 Agrichemicals

Agrichemical applications are often challenging for any analytical method because of the complexities of the sample matrices, the detection levels required, and the varieties of the compounds involved. In many cases, trace amounts of

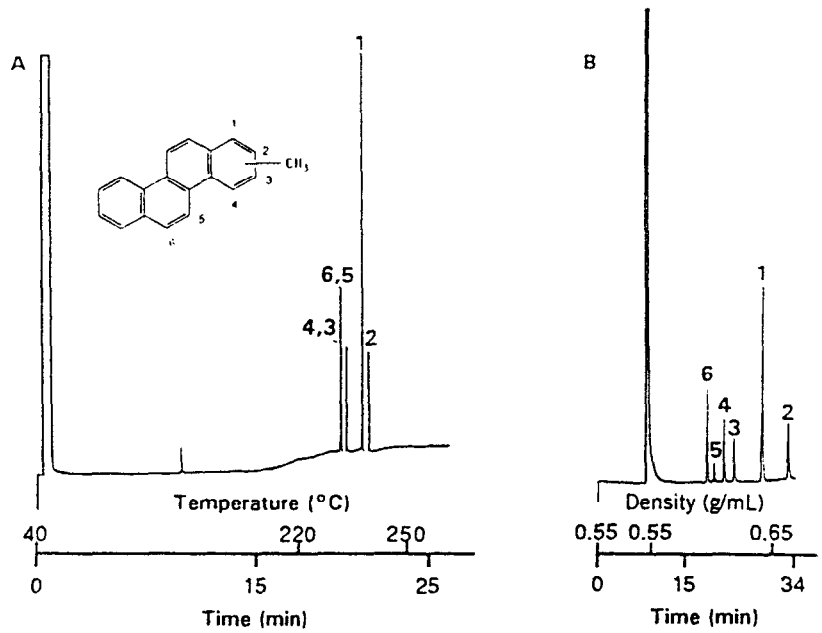

Figure 1.23 Chromatograms of six methylchrysene isomers separated on the same liquid crystalline stationary phase by (*a*) GC and (*B*) SFC. The GC conditions: 15-m × 200-μm i.d. open tubular column; 0.15-μm film thickness; temperature program from 40 to 200°C at 10°C/min, then 4°C/min to 240°C. The SFC conditions: 10-m × 50-μm i.d. open tubular column; 0.15-μm film thickness; CO_2; 100°C; density program from 0.55 to 0.70 g/mL at 0.005 g/mL/min after a 10-min isopycnic period. Peak numbers represent the positions of methyl substitution on the chrysene structure. Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, A Division of Preston Industries, Inc., Niles, IL.

the parent or its metabolite in these complex matrices must be detected at parts per billion (ppb) or parts per trillion (ppt) levels.

The most commonly used detector, the FID, has been used extensively with SFC for detecting pesticides [168, 193–197]. For relatively simple samples, short open tubular columns and rapid pressure programming achieved separations of several carbamate pesticides present in a standard mixture in less than 2 min [193, 195].

For more complex matrices, longer open tubular columns have provided the necessary separation efficiencies [194, 198]. Several sulfonylurea herbicides extracted from a ground water sample were analyzed using SFC with an FID [199]. Detection of these sulfonylurea herbicides, representing 5 ppb in a 1-L ground water sample, was easily achieved.

Linuron and diuron, extracted from soil and wheat grain using SFE, were separated from various background impurities using a microbore packed column [168]. The larger injection volumes accommodated by the microbore packed columns afforded detection limits more appropriate for trace residue analyses. Subnanogram quantities of linuron were detected, representing 10-ppb levels of the pesticide in 1-g soil samples.

Packed column SFC with a UV-absorbance detector was used to analyze sulfonylurea herbicides, as well as several additional pesticides and their

precursors on a conventional silica column [196, 197, 200]. The addition of a small amount of organic modifier to the CO_2 was required to prevent severe band broadening, to reduce retention, and to sharpen the chromatographic peaks, thereby improving the detection limits.

Recently, a UV photodiode array detector was interfaced to open tubular column SFC, allowing sensitive detection of several herbicides and pesticides [201]. Comparable sensitivities to single wavelength detection were obtained. With multichannel capability, UV detection can be used to provide additional resolution of peaks not resolved chromatographically.

Many agrichemicals contain nitrogen, phosphorus, sulfur, or halogens, making elemental specific detectors quite advantageous in reducing background interferences present in the matrix. Carbamates extracted from a parsley sample and analyzed using an NPD gave a detection limit of 1.6 ppb [198].

The flame photometric detector (FPD) is specific for phosphorus and sulfur-containing compounds. Optimization of the flame gases has led to similar detector sensitivities as experienced in GC [100]. However, with CO_2 as the mobile phase, there is an emission at the wavelength used to monitor sulfur. With density or pressure programming, the intensity of the emission increases. Therefore, at the present time, the FPD is more useful for monitoring of phosphorus-containing compounds.

The electron capture detector (ECD) has been used successfully with open tubular column SFC in the analysis of a triazole fungicide metabolite [202] and labile pesticides [107]. The sensitivity of this detector to O_2 required passing the CO_2 through an oxygen trap. In addition, elevated detector temperatures were necessary to counteract the cooling of the effluent during mobile phase decompression. An approximate detection limit of 35 ng/mL of the metabolite in solution was obtained.

The MS can serve as a universal detector for pesticide analysis. In the selected ion monitoring mode, the MS does not monitor most interferences present in the sample extracts, thereby allowing the elimination of many of the sample clean-up steps normally required. Thus, sample preparation and analysis times can be greatly reduced. Mass spectrometry detection for packed and open tubular column SFC has been used to detect acid, carbamate, and organophosphorus pesticides [143, 203–208].

Chromatographic reproducibility in terms of retention time, peak area, and peak height has been studied in detail with respect to routine analysis of agricultural samples [196, 199, 201]. Typical reproducibilities using open tubular column SFC for a series of herbicide standards were between 2 and 3% RSD based on raw peak areas and peak heights [199].

The SFE has been applied to agrichemicals for qualitative analysis, as a sample introduction method, and for quantitative analysis of trace components [168, 209–211]. As a sample introduction method, part or all of the extracted materials are introduced onto the chromatographic column for analysis and identification. Pesticides extracted from parsely [198, 210], herbicides (linuron and diuron) extracted from soil and wheat grain [168], and a herbicide extracted

from an enzyme cell culture [211] are a few examples. These qualitative experiments are useful for determining the presence of a component in a matrix, or in evaluating the potential application of SFE to a particular analysis.

The quantitative evaluation of extraction parameters has been conducted using ^{14}C-labeled herbicides, linuron, and diuron [168, 211]. The extraction effluent was collected, and recoveries were determined by liquid scintillation counting. The effects of temperature and fluid density, each independently controlled, as well as the effect of mobile phase modifier (type and amount) were evaluated. The optimized extraction conditions were compared to conventional methodologies previously used. Supercritical fluid extraction for almost 1 h produced comparable or better extraction efficiencies than the conventional procedures, which required up to 3 days of labor intensive effort (see Table 1.15). For the compounds examined, modifying the CO_2 with small amounts of organic solvents, such as methanol or ethanol, was necessary for quantitative recoveries.

6.4 Explosives and Propellants

Douse [212] showed that by using SFC, nonvolatile and/or thermally labile explosives could be separated from a complex mixture without tedious sample clean-up. Griest and co-workers [213] showed a separation of high explosives using packed-column SFC and UV detection.

To detect trinitrotoluene (TNT) and other explosives at trace levels, mass spectrometry is the most powerful on-line detector. Minimum detectable quantities of 1–5 ng for full scan operation and below 100 pg for selected-ion-monitoring after electron impact ionization are common. With negative ion chemical ionization (NICI), 100-fold improvements in detection limits can be obtained. Figure 1.24 shows the NICI SFC/MS analysis of 10 pg of TNT and other related compounds [214].

Propellant analysis is another important application of SFC. The determination of stabilizers in the propellant is important for the prediction of the safe "shelf life" of the propellant. A major stabilizer used is diphenylamine, which

Table 1.15 Comparison of Recoveries of Diuron and Linuron from Soil by SFE and Classical Extraction[a]

Compound	*Method*	*Time*	*Modifier Volume*	*Percent Recovery*
Diuron	SFE	35 min	200 μL MeOH	99
	Classical	3 days	>1 L solvent	96
Linuron	SFE	50 min	500 μL EtOH	95
	Classical	3 days	>1 L solvent	82

[a]Taken from [168].

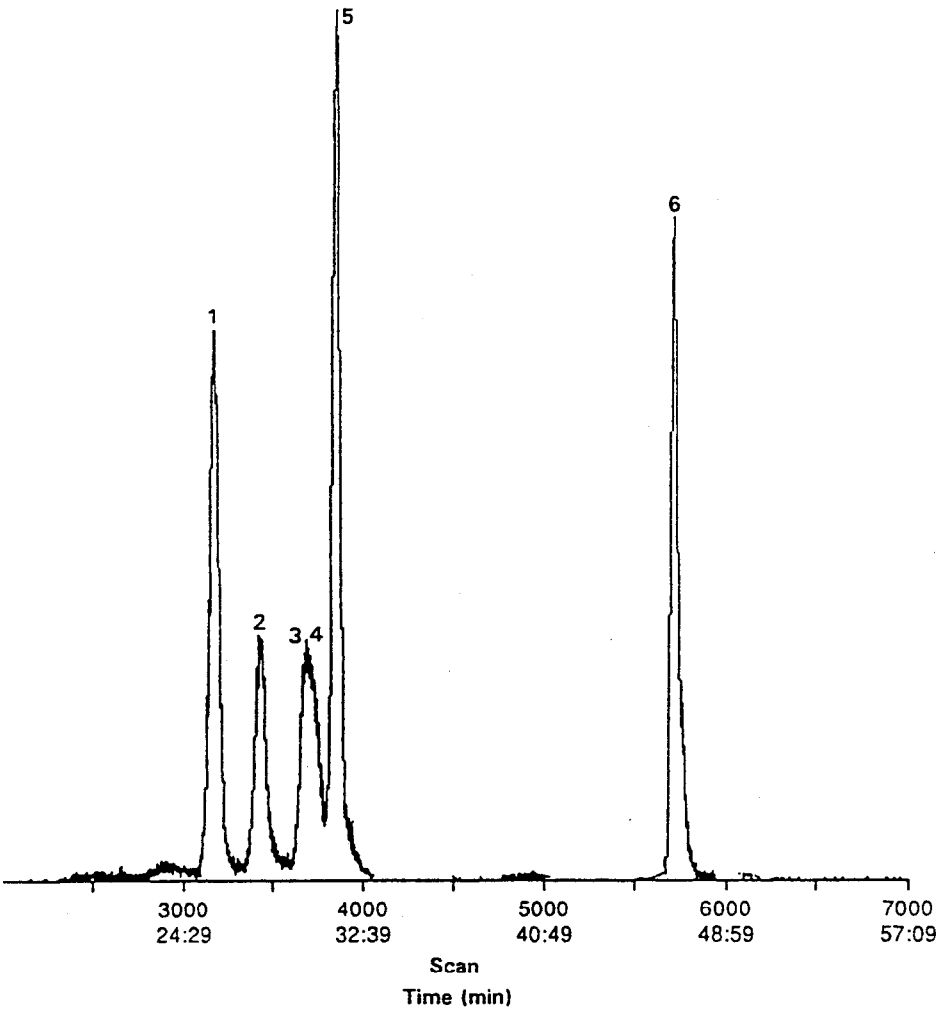

Figure 1.24 An SFC/MS total ion chromatogram of TNT and related compounds. Conditions: 10-m × 50-μm i.d. open tubular column, poly(5%phenyl)methylsiloxane (d_f = 0.15 μm); CO_2; 100°C; density program from 0.3 to 0.75 g/mL at 0.0075 g/mL/min after a 10-min isopycnic period; 0.01 ng each by direct injection; methane–NICI–MS (selected ion monitoring). Peak identifications: (1) 2,6-dinitrotoluene, (2) 2,4-dinitrotoluene, (3) 4-nitrophenol, (4) 1,3,5-trinitrotoluene, (5) 2,4,6-trinitrobenzene, and (6) 1-nitropyrene.

is capable of combining with the nitrogen oxides liberated during decomposition of the propellant. This leads to production of nitro- and nitroso-derivatives of diphenylamine. In routine GC analysis, these derivatives cannot be analyzed because they decompose to diphenylamine. Also nitroglycerin, the major component of most propellants, cannot readily be determined by GC without some thermal degradation.

Recent articles by Ashraf-Khorassani and Taylor [215, 216] showed that SFC analysis of propellants and stabilizers does not lead to decomposition of the nitro or nitroso derivatives of diphenylamine, but can produce complete separation of these derivatives. Also, chromatographic analysis of nitroglycerin was achieved with no decomposition. Figure 1.25 shows the analysis of a propellant formulation using a packed capillary column [217].

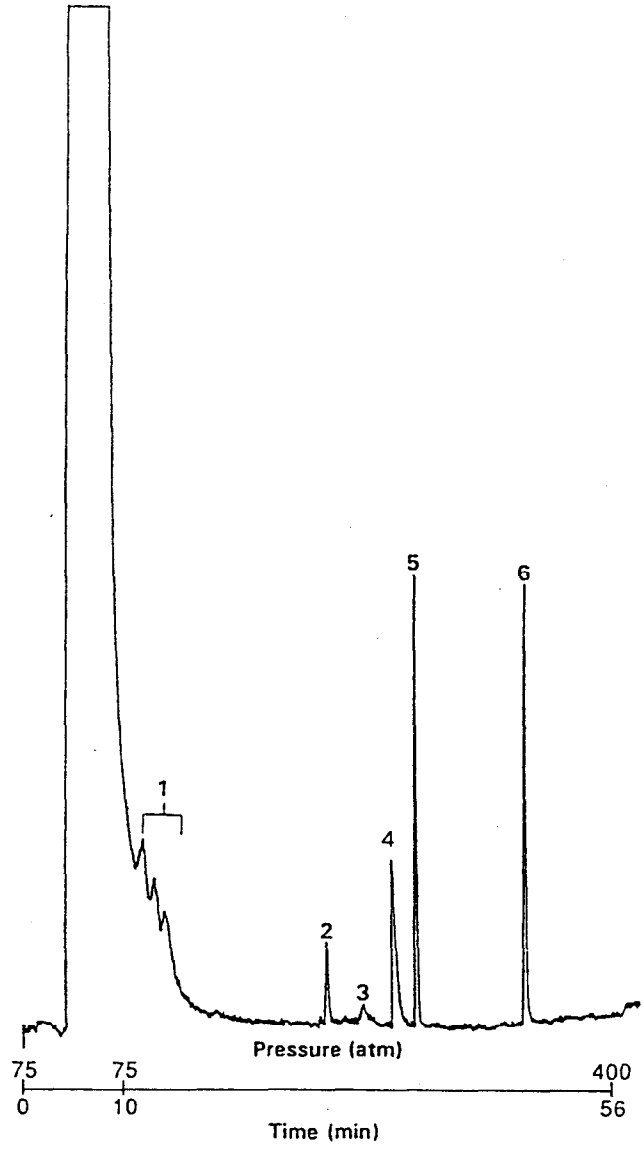

Figure 1.25 An SFC chromatogram of a double-base propellant. Conditions: 25-cm × 250-μm i.d. packed column, poly(50% octyl)methylsiloxane bonded silica, 5-μm particles; CO_2; 120°C; pressure program from 75 to 400 atm at 7 atm/min after a 10-min isobaric period; FID. Peak identifications: (1) candelilla wax, (2) 2-nitrodiphenylamine, (3) resorcinol, (4) nitroglycerin, (5) triacetin, and (6) di-*n*-propyl adipate. Reprinted with permission from K. M. Payne, B. J. Tarbet, J. S. Bradshaw, K. E. Markides, and M. L. Lee, *Anal. Chem.*, **62**, 1379 (1990). Copyright © 1990 American Chemical Society.

6.5 Drugs and Pharmaceuticals

In the area of drug analysis, SFC may be regarded as a complementary technique to the more traditional techniques such as immunoassays, LC, GC, and coupled chromatography/MS [218]. Potential applications of SFC in substance abuse testing have been demonstrated. Crowther and Henion [219] reported on the use of a polar packed column in SFC with MS detection for the

analysis of cocaine. Fast analysis was achieved at high linear velocity without significant loss in efficiency. The high efficiency also enabled higher sensitivity than was possible by LC/MS. In addition, it was shown how SFC/MS is preferred over GC/MS for the analysis of thermally unstable polar drugs.

Tetrahydrocannabinol and six common metabolites were resolved on a nonpolar open tubular column, but a more selective column was recommended for baseline separation of all metabolites [220]. Acid extracts of heroin samples for fingerprint information have been analyzed using an efficient and inert open tubular column with universal flame ionization detection [221]. In addition, a series of opiates were analyzed successfully using open tubular column SFC with ion mobility detection without the need for derivatization [222].

A preliminary study of the analysis of phenobarbital by open tubular column SFC provided an examination of the technical feasibility and results compared to an established routine clinical fluorescence polarization immunoassay [223]. Figure 1.26 shows the SFC chromatograms of extracts of a drug-free serum and a patient serum. Precision studies showed that within-run and day-to-day mean phenobarbital concentrations were 25.8 ($n = 5$) and 23.8 ($n = 13$) mg/L, respectively, compared to 25.8 mg/L estimated by fluorescence polarization immunoassay. The corresponding coefficients of variation were 6.9 and 12.9%. The precision must be improved to the clinically accepted limits of less than both 5 and 10% for within-run and day-to-day coefficients of variation, respectively.

The possibility of analyzing underivatized prostaglandins by open tubular column SFC using neat CO_2 as the mobile phase and the universal FID [20] for detection was recognized early. In biological samples, prostaglandins are present only in trace amounts, making it preferable to use solvent elimination sample introduction methods. Underivatized prostaglandins have been analyzed from water solutions by Koski and co-workers [224] using SFE injection.

Berger and Deye [225] demonstrated the use of tetrabutylammonium hydroxide in methanol as an additive to supercritical Freon 23 mobile phase for successful elution of promazine and thioridazine on a packed column. Benzodiazepines can be eluted with pure CO_2 if a well deactivated and short column is used as demonstrated by Koski [226], while 5–13% methanol modifier in CO_2 was recommended for packed column analysis [227]. Medium polarity stationary phases were used for both column types.

For basic drugs of higher polarity and primary amine functionality, it is no longer possible to use CO_2 as the mobile phase. An initial feasibility study of the use of ammonia as the mobile phase with open tubular columns gave rapid elution of a primary amine even when injected as a hydrochloride salt [17].

The steroid hormones are members of a class of numerous and extremely similar molecules with widely divergent physiological effects. A recent report described the use of SFC with selective thermionic detection [104] and Fourier transform–infrared (FT–IR) [228] for the analysis of a number of steroid hormones. An alternative detection approach was introduced for several steroids based on thiophosphinic ester derivatization [104]. The thiophosphinic

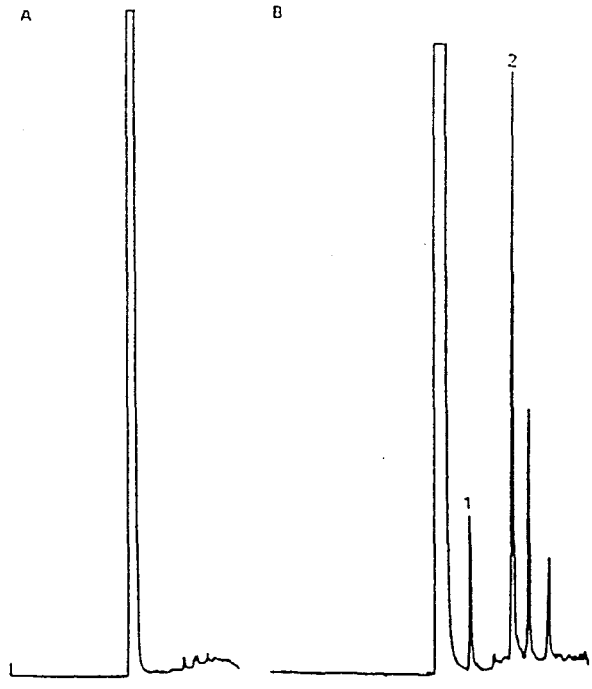

Figure 1.26 The SFC chromatograms of (*a*) a drug-free serum extract and (*b*) a serum extract of a patient medicated with phenobarbital. Conditions: 10-m x 50-μm i.d. open tubular column, polymethylsiloxane stationary phase; CO_2; 120°C; density program from 0.25 to 0.75 g/mL at 0.06 g/mL/min. Peak identifications: (1) internal standard and (2) phenobarbital. Reprinted by courtesy of Marcel Dekker, Inc.

group, when reacted with the steroyl hydroxyl groups, introduces specificity for phosphorus selective detection at the femtogram level.

Open tubular column analysis of underivatized anabolic steroids, dexamethasone and betamethasone, the adrenocortical prednisone steroids, and the cortical steroids and hydrocortisone was demonstrated [220]. The SFC separation could be performed at a low enough temperature to avoid epimerization between the betamethasone and dexamethasone forms.

The resolution of steroid isomers was improved significantly using a liquid crystalline polysiloxane stationary phase [21] when compared to results obtained using other stationary phases. Figure 1.27 shows the separation of androstane diols and triols where the retention mechanism is related to solute molecular shape rather than to properties such as volatility and polarity.

6.6 Polymers

Of the many fields in which SFC has attracted considerable attention, the field of polymer analysis is one of the most important. This is focused on several different aspects:

1. Determination of low molecular mass components in polymers, such as residual monomers and solvents; oligomers; and additives like stabilizers,

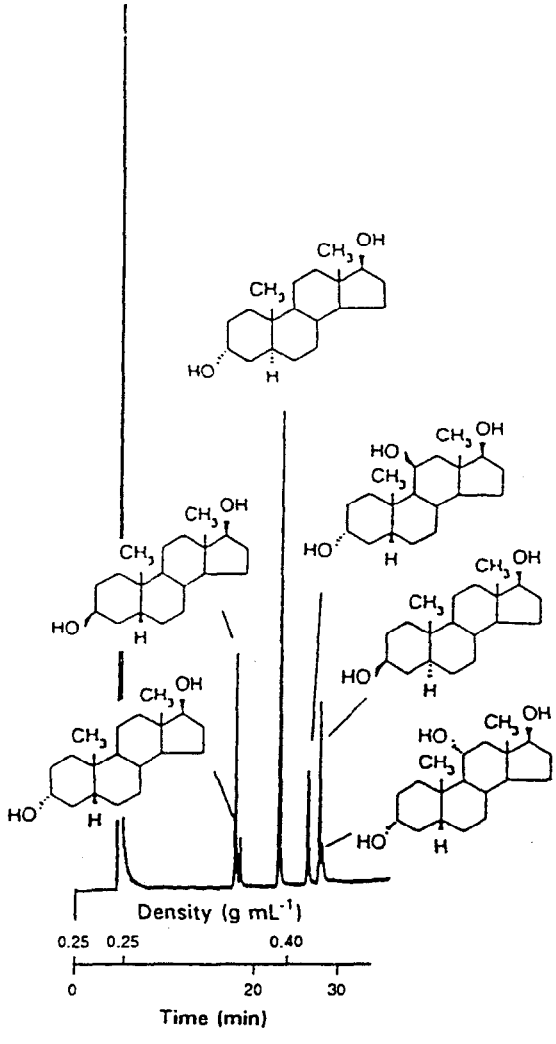

Figure 1.27 An SFC chromatogram of four androstane diols and two androstane triols separated on a liquid crystal column. Conditions: 6-m × 50-μm i.d. open tubular column (d_f = 0.15 μm); CO_2; 120°C; density program from 0.25 to 0.50 g/mL at 0.0075 g/mL/min after a 5-min isopycnic period; FID. Reprinted with permission from H-C. K. Chang, K. E. Markides, J. S. Bradshaw, and M. L. Lee, *J. Microcol. Sep.*, **1**, 131 (1989).

plasticizers, and surface treatments. For the determination of these compounds in a high-polymer matrix, the combination of SFE and SFC is very effective.

2. Separation of homologous series (i.e., oligomers) of low molecular mass. For such samples, the determination of the molecular mass distribution is of interest.
3. Assay of parallel homologous series. Such information may be of special interest for industrial purposes (as "fingerprint" information).
4. Purity tests for chemicals such as monomers and reactive oligomers and prepolymers with respect to purity, batch-to-batch reproducibility, fingerprint information, and so on.

Styrene oligomers have been the most intensively studied oligomers by SFC. These oligomers were chosen for the first pressure programmed separation reported in the literature [229]. Furthermore, styrene oligomers have been used for testing the applicability of multiple gradients [230, 231]. Finally, use was made of styrene oligomers for evaluating the dependence of retention on

temperature, pressure, and density of the mobile phase, and on the pore diameter [232] of the stationary phase. For minimizing the time requirements for such fundamental studies, styrene oligomers were replaced by aromatic compounds, which were shown to resemble them with respect to their retention behavior [233].

Like the other vinyl arene oligomers, styrene oligomers have been studied primarily using packed columns. Gradient techniques that have been used for the separation of these oligomers include pressure/density gradients, composition gradients, and temperature gradients. With both pressure and composition gradients, up to approximately 70 homologs can be separated [234, 235], which corresponds to a molecular mass range of 7300. With a proper combination of mobile and stationary phases, the separation of stereoisomers was possible [236].

t-Butyldimethylsilyl (TBDMS) derivatives of acrylic acid oligomers, which contain one carboxylic acid side group per repetitive unit, were successfully chromatographed by Pinkston [237] on an open tubular column coated with polymethylsiloxane by means of neat CO_2 and pressure programming. Separation was achieved for some 20 homologs; higher molecular mass species remained unresolved. Methyl methacrylate oligomers, as obtained from radical oligomerization, were chromatographed with a composition gradient on a column packed with a silica stationary phase [238].

Oligomers with the general repeating unit, —O—R— are very readily separated by means of supercritical CO_2. While there are only single reports mentioning the separations of oxymethylene ($R = CH_2$) [239] and oxyphenylene ($R = C_6H_4$) [240] oligomers, separations of oxyethylene ($R = CH_2—CH_2$) oligomers are numerous. In the majority of cases, the separations of the poly(ethylene oxide) or poly(ethylene glycol) samples have been carried out by means of open tubular columns using pressure/density programming with a CO_2 mobile phase. The polyethylene oxides, which are frequently used as nonionic detergents/surfactants, are produced by ethoxylation of fatty alcohols. When such alcohols are technical grade products, they may contain more than only one hydroxyl component; this leads to the formation of parallel series. The SFC chromatogram of such a product is shown in Figure 1.28 [241], where the two main series originating from ethoxylation of tetradecanol and pentadecanol are seen along with smaller additional series.

Other products separated by SFC have been obtained by esterification of polyglycols with fatty acids. The separation of such a product, obtained by condensation of stearic acid and poly(ethylene glycol), gave two main series in the chromatogram that can be assigned as chains bearing one or two stearate ends, respectively. A similar distribution has been reported for a polymer additive composed of esterified poly(ethylene glycol) [242]. Additional industrial samples based on poly(ethylene oxide), including an ethoxylated amine [243], and their separation by SFC have been described [243–245].

Resolution of up to 80 homologs of polymethylsiloxanes and poly(phenyl)methylsiloxanes has been achieved [242]; in Figure 1.29, polymethylsi-

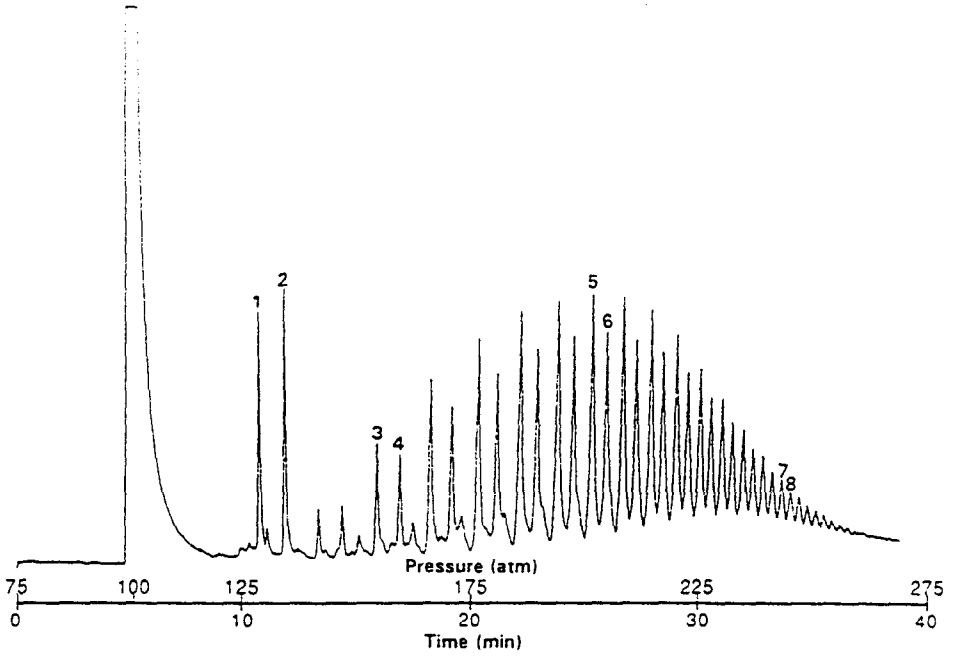

Figure 1.28 An SFC chromatogram of ethoxylated alcohols (Neodol 45-7T) with straight-chain C_{14} and C_{15} alcohols spiked as retention standards. Conditions: 10-m × 50-μm i.d. open tubular column, polymethylsiloxane stationary phase (d_f = 0.2 μm); CO_2; 120°C; pressure program from 100 to 275 atm at 5 atm/min after a 5-min isobaric period; FID (300°C). Peak identifications: (1) *n*-C_{14} alcohol, (2) *n*-C_{15} alcohol, (3) $C_{14}E_2$, (4) $C_{15}E_2$, (5) $C_{14}E_7$, (6) $C_{15}E_7$, (7) $C_{14}E_{15}$, and (8) $C_{15}E_{15}$. Reprinted with permission from J. D. Pinkston, D. J. Bowling, and T. E. Delaney, *J. Chromatogr.*, **474**, 97 (1989).

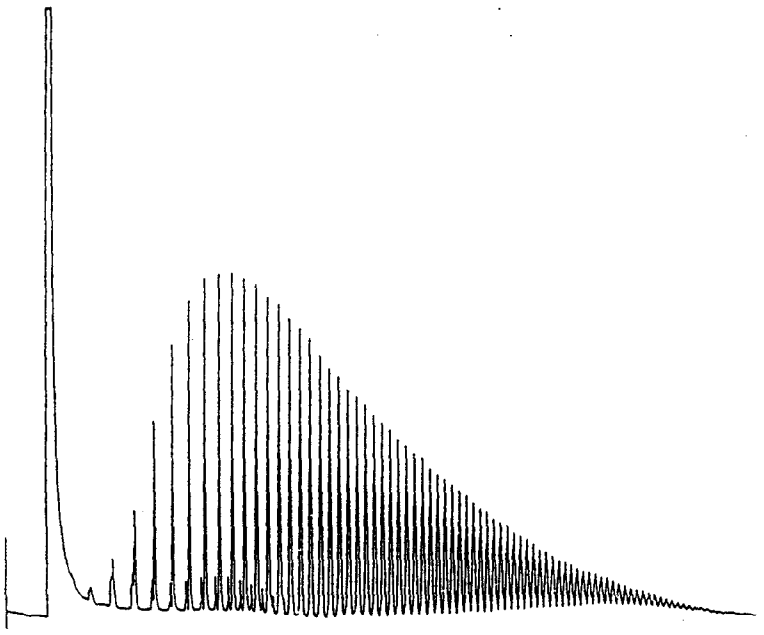

Figure 1.29 An SFC chromatogram of dimethylsiloxane oligomers. Conditions: 20-m × 50-μm i.d. open tubular column, polymethylsiloxane stationary phase; CO_2; 120°C; asymptotic density program; FID. Reprinted with permission from K. D. Bartle, M. P. Burke, A. A. Clifford, I. L. Davies, J. P. Kithinji, M. W. Raynor, G. F. Shilstone, and A. Williams, *Eur. Chromatogr. News*, **2**(5), 12 (1988).

loxane and poly(phenyl)methylsiloxane oligomers have been chromatographed up to a molecular mass of 8000, corresponding to approximately 60 homologs [243]. Siloxane oligomer separations have been used for testing injection techniques [76] and simultaneous density and temperature programming [246, 247].

A variety of additional oligomeric products have been analyzed by SFC. Different perfluorinated materials, such as tetrafluoroethylene oligomers [248], a fluorinated surfactant [248], and a "functionalized perfluoropolyether" [249] were chromatographed by means of a pressure gradient. In addition, different end- or side-group reactive oligomers should be mentioned, such as ethoxy acrylate oligomers [76, 250], allyl ethoxylate oligomers [237], and a Novolac product with epoxy side groups [236]. Other products of technical interest are, for example, different types of waxes and hydrocarbon mixtures.

A special analytical problem is the identification and determination of polymer additives. Such additives may be single components, or they may be composed of a homologous series themselves. In an increasing number of publications, separations of polymer additives have been mentioned [242, 245, 251–257]. For the analysis of additives, SFE proves of great advantage [252].

As mentioned above, SFC can be a useful analytical technique for checking lot-to-lot reproducibility of polymer manufacturing. This has been demonstrated [247] for two "polyglycol" lots from the same manufacturer. One of these lots did not perform correctly for the user. The reason for this was found in the difference in the molecular mass distributions of the two products. One study reported the use of SFC for process control monitoring of an epoxy resin manufacturing process [251].

6.7 Lipids

The separation of fatty acids, and mono-, di-, and triglycerides has been studied extensively with open tubular column SFC. Supercritical CO_2 is an excellent solvent for fats, oils, fatty acids, and most of their derivatives. As a result, triglycerides and their components are used often to demonstrate improvements in SFE and open tubular column SFC techniques.

Schwartz and co-workers [258] used an interesting combination of modifiers (0.3% formic acid and 0.15–0.18% water) for FID detection with packed column SFC. For the most difficult separations, open tubular column SFC generally provides the best resolution, although there is some disagreement on this point [259, 260]. Sandra and co-workers [260] compared separations using high temperature open tubular column GC and open tubular column SFC of a variety of lipid samples including milk chocolate, palm kernel oil, and several mixtures consisting of mono-, di-, and triglycerides. In most cases, GC compared favorably with open tubular column SFC, although SFC was favored for (1) certain compounds with molecular mass above approximately 1400, (2) phospholipids, (3) mixtures that require the use of selective phases like cyanopropyl or polyethylene glycol (with temperature limitations of 250–280°C), and (4) samples for which fraction collection is desired. As an example,

fatty acid hydroperoxides decompose well below the temperatures required to volatilize these thermally sensitive analytes. Consequently, either SFC or LC must be used to separate the positional isomers of unsaturated fatty acid hydroperoxides [261].

For separations of fatty acids or their derivatives (e.g., fatty acid methyl esters) based on the number of carbon atoms, nonpolar stationary phases, [e.g., polymethylsiloxanes or poly(phenyl)methylsiloxanes] are sufficient. However, separations of triglycerides or fatty acids of the same carbon number, but with a different number of double bonds, are usually better accomplished on more polar stationary phases, such as the cyanopropyl-containing or the polyethylene glycol stationary phases. An excellent example of the selectivity provided by the more polar stationary phases was reported by Richter and co-workers [198].

6.8 Carbohydrates

During the last two decades, there has been an increasing interest in the details of carbohydrate structure. Glycoconjugates, detected in plant and animal tissues of all types, are associated with countless cellular processes that have aroused and engaged the interest of many biological researchers. Our structural understanding of these materials remains primitive when contrasted to the analytical expertise we enjoy with proteins and nucleic acid biopolymers.

Using trimethylsilyl derivatization to make oligosaccharides less polar, CO_2 as a mobile phase, and open tubular columns [73, 262], Chester and Innis [263] demonstrated the separation of a series of glucose oligomers up to degree of polymerization (DP) = 18 (Figure 1.30). The remarkable column resolution was apparent from the baseline separation of each incrementing monomer, and demonstrated again within each oligomer by their α- and β-anomeric separation. The molecular mass of the higher oligomers exceeded 6000, a mass range well above anything demonstrated by GC. Silylation served to induce solvent miscibility with the less polar CO_2.

Kuei and co-workers [264] evaluated SFC for the characterization of glycolipid samples following permethylation. In this report, several neutral and acidic glycolipid samples and two tissue mixtures were investigated. One sample of considerable complexity was an extract from brain tissue known to consist of gangliosides (neuraminic acid containing). This material was permethylated and chromatographed on an open tubular column.

6.9 Industrial Chemicals

Carboxylic acids are important natural products and intermediates for a wide variety of compounds. Their highly polar nature and tendency to form hydrogen bonds usually require derivatization in GC techniques. Neat supercritical CO_2 and N_2O have sufficient solvent strength to dissolve and transport the C_1–C_{30} free acids [265] in concentrations typically associated with open tubular column SFC. Figure 1.31 shows the separation of 15 aromatic acids from benzoic to *trans-p*-hydroxycinnamic acid [242].

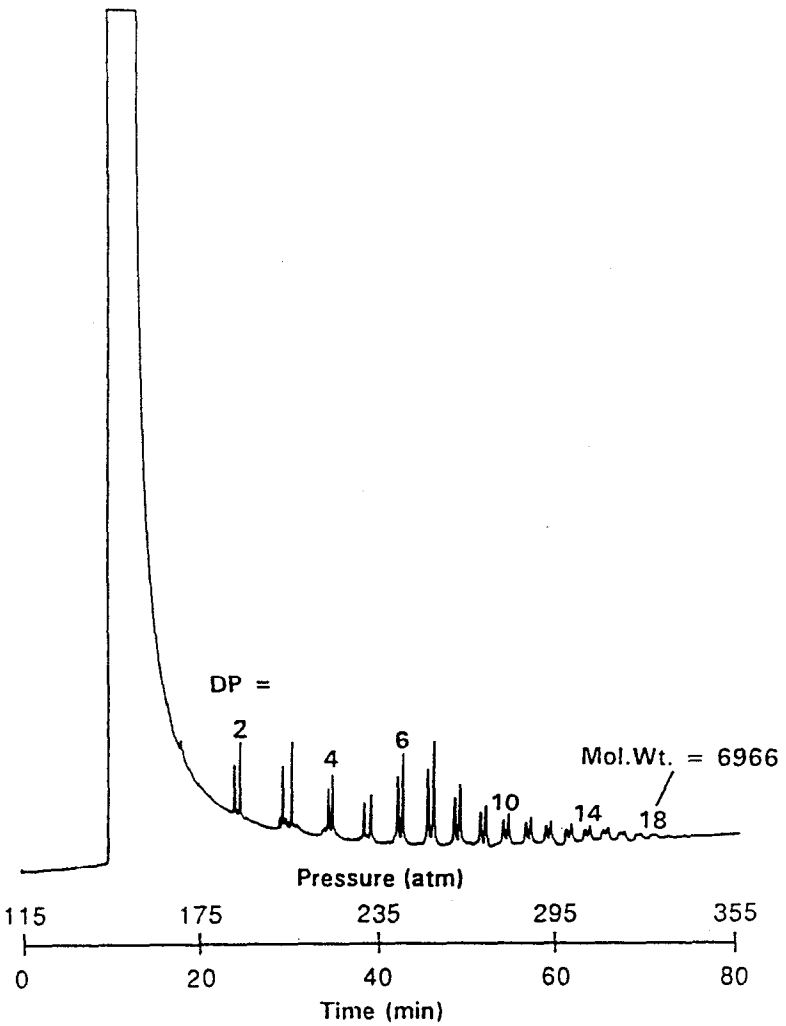

Figure 1.30 An SFC chromatogram of silylated Maltrin 100. Conditions: 10-m × 50-μm i.d. open tubular column, poly(5% phenyl)methylsiloxane stationary phase; CO_2; 89°C; FID. Reprinted with permission from T. L. Chester and D. P. Innis, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, **9**, 209 (1986).

Amines differ strongly in their basicities and, therefore, reactivities with silanol surface groups or acidic mobile phases. An extensive study of the reactivity of CO_2 with amines led to the conclusion that amines with pK_b values lower than approximately 9 react with supercritical CO_2 [266, 267]. The formation of insoluble salts and decomposition products was observed for some compounds, while immiscibility and variable solubility were observed for others.

With respect to chromatographic behavior, several guidelines have been deduced from systematic experiments [268]. Aliphatic tertiary amines, such as triethylamine, tributylamine, trihexylamine, and trioctylamine, can be eluted from a polymethylsiloxane stationary phase with good peak shapes using CO_2 as mobile phase. To analyze strongly basic amines, two approaches are feasible: (1) conversion to trifluoroacetyl or other acetyl derivatives [269] or (2) direct

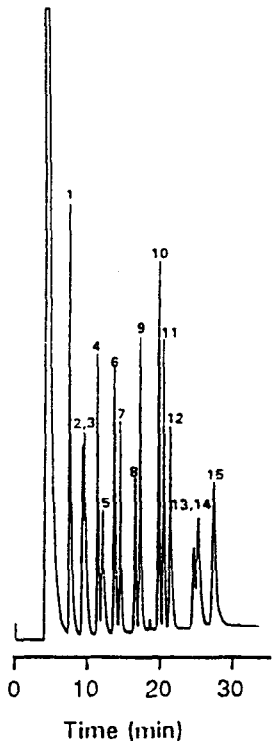

Figure 1.31 An SFC chromatogram of aromatic acids. Conditions: 10-m × 50-μm i.d. open tubular column, poly(oligoethylene oxide)-methylsiloxane stationary phase; CO_2; 100°C; density program from 0.05 to 0.76 g/mL at 0.015 g/mL/min after a 5-min isopycnic period; FID. Peak identifications: (1) benzoic acid, (2) cinnamic acid, (3) 4-chlorobenzoic acid, (4) 3,5-dimethoxybenzoic acid, (5) chlorocinnamic acid, (6) vanillic acid, (7) 3,4-dimethoxycinnamic acid, (8) syringic acid, (9) ferrulic acid, (10) sinapic acid, (11) *p*-hydroxyphenylpropionic acid, (12) *p*-hydroxyphenylacetic acid, (13) *p*-hydroxybenzoic acid, (14) *cis-p*-hydroxycinnamic acid, (15) *trans-p*-hydroxycinnamic acid. Reprinted with permission from K. D. Bartle, M. P. Burke, A. A. Clifford, I. L. Davies, J. P. Kithinji, M. W. Raynor, G. F. Shilstone, and A. Williams, *Eur. Chromatogr. News*, **2**(5), 12 (1988).

analysis of the primary alkyl amines by employing inert mobile phases such as SF_6 [270] or N_2O and very well deactivated columns [269]. Unless mixed with modifiers, both fluids exhibit low solvent strength and are limited to low molecular mass and monofunctional compounds [271].

Isocyanates are reactive, and the thermal labilities of many isocyanate derived products makes their analysis difficult. The types of isocyantes used as bulk chemicals for polyurethane manufacturing range from low molecular mass to medium molecular mass polymers, and include monomeric and polymerized derivatives such as ureas, dimers and trimers, blocked isocyanates, and prepolymers of isocyanates and polyols.

Open tubular column SFC [272] can overcome the above problems, provided the mobile phase, instrument components, and solvents are kept meticulously free of water and alcohols. Typical chromatograms of 4,4'-diphenylmethane diisocyanate samples are shown in Figure 1.32. Columns with 100-μm i.d. and film thicknesses of 0.25–0.5 μm were used to optimize sample capacity for the inherent minimum detection limits of the FID. Short column lengths were used to decrease the analysis time.

6.10 Foods and Flavors

Gere and co-workers [273, 274] used a column packed with 5-μm particles of octadecyl-bonded silica to separate vitamins A, E, and D, using a methanol/CO_2 mobile phase gradient (0.5% for 7.5 min, to 1.0% in 2.5 min). However, addition of modifiers is not necessary for the elution of lipid-soluble vitamins. White and co-workers [92] reported the separation of a vitamin mixture by SFC using a

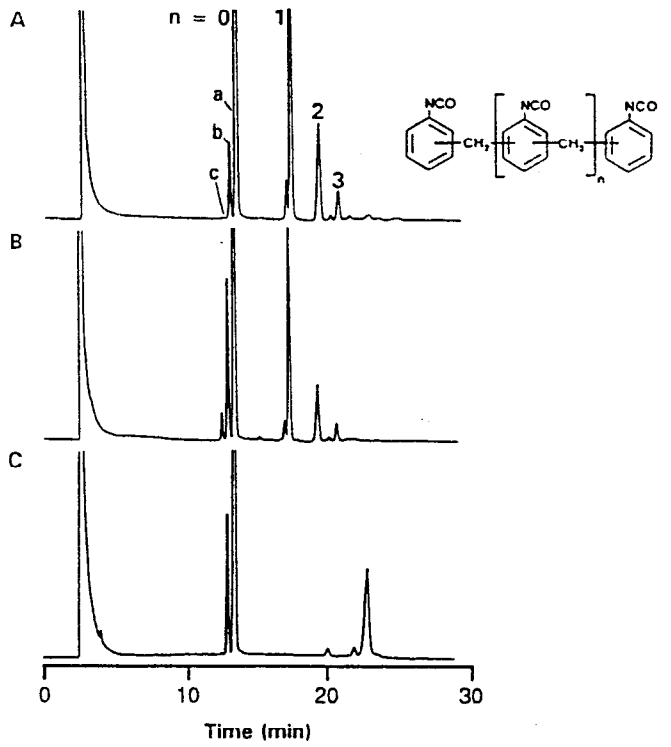

Figure 1.32 The SFC chromatograms of liquid diphenylmethane diisocyantates (MDI). The first grouping of peaks (a–c) corresponds to the MDI in the isomers polymeric structure $n = 0$; the numbered peaks refer to the oligomer series of $n = 1-3$: (*A*) Technical-grade MDI, (*B*) Desmodur VL, (*C*) Isonate 143L. Conditions: 5-m × 100-μm i.d. open tubular column, polymethylsiloxane stationary phase ($d_f = 0.5\ \mu m$); CO_2; 100°C; density program from 0.2 to 0.7 g/mL at 0.03 g/mL/min after a 5-min isopycnic period; FID. Peak identifications: (a) 4,4′-MDI, (b) 2,4′-MDI, (c) 2,2′-MDI. Reprinted with permission from S. M. Fields, H. J. Grether, and K. Grolimund, *J. Chromatogr.*, **472**, 175 (1989).

cross-linked Carbowax 20M open tubular column. Excellent reproducibility of peak areas and retention times was achieved.

An unusual application of SFE and SFC for wheat germ tocopherols has been reported [275]. Supercritical fluid extraction was used to extract the lipid components of the wheat germ, and this was followed by preparative SFC to increase for evaluation the tocopherol concentration in the lipid extract. Multichannel UV detection allowed independent monitoring of lipid and tocopherol elution to optimize this difficult separation.

Nitrosamines are carcinogenic compounds that are formed when amines and nitrites react. Amines are ubiquitous in most foods; nitrites are added to preserve the foods. The use of selective detectors with SFC makes it possible to analyze complex matrices containing nitrosamines without extensive cleanup and prefractionation steps [276].

Essential oils from citrus fruits contain terpenes, sesquiterpenes, oxygenated compounds, and nonvolatile residues. The terpenes contribute little to the flavor or fragrance of the oil. Since the terpenes are mostly unsaturated compounds, they can decompose from heat, light, and oxygen to compounds with unde-

sirable flavors and odors. The major components of citrus fruit oils, terpenes, and sesquiterpenes, have been analyzed by several research groups with SFE/GC [169], SFE/SFC [160], and SFC/FT–IR (Figure 1.33) [277–279]. The advantage of FT–IR over MS is the possibility to distinguish isomers [277].

6.11 Natural Products

Berry and co-workers [206, 280, 281] investigated a wide variety of natural products, including indol and ergot alkaloids and steroids. Analytical SFC was also applied in conjunction with thermospray (filament-on mode) and electron ionization (EI) MS. Thermospray ionization generated molecular mass information on the eluting components; however, peak intensities did not correspond to the actual ratios of the alkaloids in the mixture.

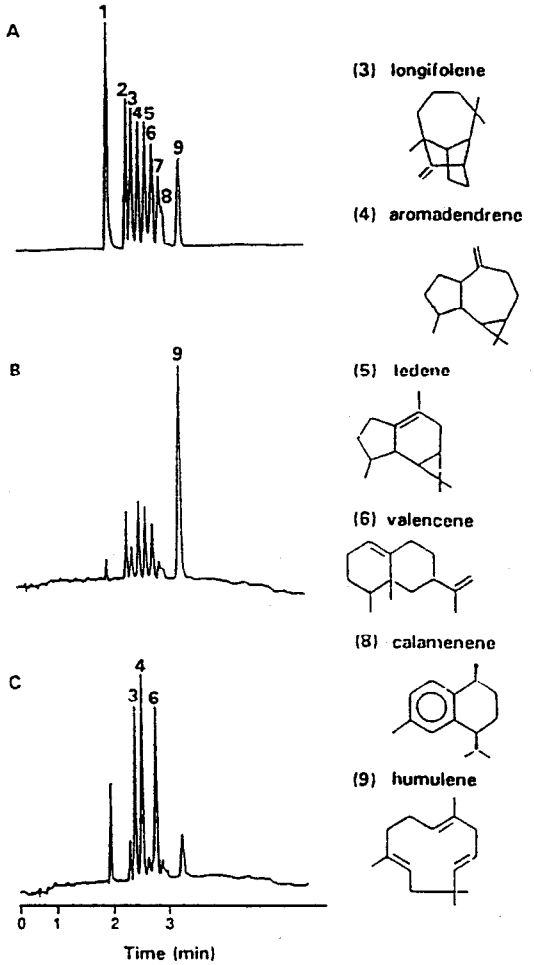

Figure 1.33 The SFC/FTIR chromatograms of a mixture of eight sesquiterpene hydrocarbons: (*a*) Gram-Schmidt total IR, (*b*) 957–987-cm^{-1} spectral window, and (*c*) 849–895-cm^{-1} spectral window. Conditions: two 250 x 4.6-mn i.d. packed columns in series, Nucleosil 100 and Spherisorb 5-μm particles; CO_2; 40°C; 130 atm. Peak identifications: (1) hexane, (2) longicyclene, (3) longifolene, (4) aromadendrene, (5) ledene, (6) valencene, (7) *trans*-calamenene, (8) *cis*-calamenene, and (9) humulene. Reprinted with permission from P. Morin, H. Pichard, H. Richard, M. Caude, and R. Rosset, *J. Chromatogr.*, **464**, 125 (1989).

Unlike the glycerol bound fatty acids, which are the structural elements of most biological membranes, a select group of organisms known as archaebacteria, contain isopranylglycerol ether lipids [282]. These compounds are so unusual that they serve as chemotaxonomic markers for these organisms. The study of their occurrence and transformations in organic sediments has greatly advanced the understanding of geochemical processes.

Separations of lipids from some interesting microbial sources have been reported [283, 284]. Glycerol tetraethers were extracted from methanogenic thermophilic bacteria. The archaebacteria were isolated from 2000 m below the surface near a hydrothermal vent in the Gulf of California. The lipids differed in the number of cyclopentane rings present in the isopranyl side chains. Separation had been difficult to achieve with LC, TLC, or GC, and direct analysis was only possible with open tubular column SFC.

A different class of fungal components that has been studied by SFC is the tricothecene mycotoxins. The SFC studies of these materials were reported in two papers using open tubular column SFC with MS detection [285, 286]. The earlier report [285] demonstrated separations of a variety of mycotoxins ranging in molecular mass from 296 to 532. The very large roridin A and E structures were not resolved, but they could be identified by single ion plots. It was also demonstrated that the same components could be analyzed in less than 5 min with little loss in column resolution by rapid programming. The MDQ was approximately 1 pg with this system using ammonia chemical ionization (CI).

Another class of bacterial components of considerable interest are the ubiquinone structures isolated from the causative agent of "Legionnaire's Disease," *Legionnella pneumophila.* Ubiquinones are a class of isoprenoid quinones, which includes Coenzyme Q-12 (a major link in the electron-transport chain), which are ubiquitous in biological tissues. Excellent separations of these materials have been reported using octadecyl silane (ODS) reversed-phase columns with methanol-doped CO_2 as a mobile phase (Figure 1.34) [287].

Terpenes are a diverse group of compounds that are derived from isoprene precursors. They are found in most higher plants and are largely responsible for the characteristic odors. Terpenes are composed of two (monoterpenes) to eight isoprene units (carotenoids) varying in the number of double bonds and cyclic structural components. The separation of sesquiterpenes by SFC with FT–IR detection was demonstrated by Morin and co-workers [277, 279, 288]. An important benefit of this technique is the identification of sample components by their IR spectra.

Carotenoids are tetraterpenoids that occur in all photosynthetic and some nonphotosynthetic organisms. All oxygen-free, and several hydroxyl-, keto-, methoxy-, and acetyl-substituted carotenoids are soluble in supercritical CO_2 and are amenable to SFC. The separation of α-carotene and β-carotene by SFC was reported by Giddings and co-workers [289]. Gere [274] used a 3-μm particle C_{18} reverse-phase column with EtOH/CO_2 as the mobile phase to separate lycopene and α- and β-carotene.

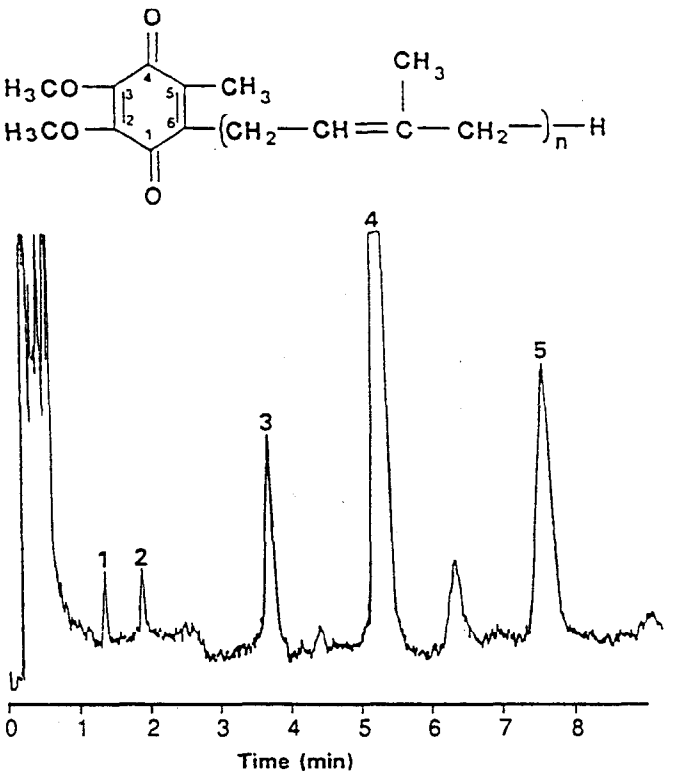

Figure 1.34 An SFC chromatogram of ubiquinones (Q-*n*) from *Legionella pneumophila*. Conditions: 10-cm × 4.6-mm i.d. packed column, C_{18} bonded silica, 3-μm particles; 1.5% methanol modified CO_2; 40°C; average column pressure (232 atm); pressure drop (69 atm); 0.87 g/mL average mobile phase density, FID. Peak identificatins of ubiquinones: (1) Q-8, (2) Q-9, (3) Q-11, (4) Q-12, (5) Q-13. Reprinted with permission from D. R. Gere, *Science*, **222**, 253 (1983). Copyright © 1983 by AAAS.

The real advantage of SFC is the high efficiency by which complex mixtures from natural sources can be separated. Using cross-linked poly(cyanopropyl)-methylsiloxane and Carbowax open tubular columns, Frew and co-workers [290] obtained good separation of β-carotene, echinenone, canthaxanthin, astacene, and fucoxanthin; however, the more polar carotenoids did not elute.

6.12 Metal Chelates and Organometallic Compounds

The analytical capabilities of SFC are usually applied to organic compounds, even though the first reported SFC separation in 1962 was of nickel porphyrin complexes [30]. Porphyrins are compounds derived from chlorophyll and are found in sediments as desoxophylloerythroetioporphyrin and mesoetioporphyrin. Fossil porphyrins are chelated with nickel or vanadium. After demineralization, they can be separated by SFC with CO_2 as the mobile phase [291]. Vanadyl porphyrins were analyzed on poly(30% biphenyl)methylsiloxane coated open tubular columns using 8.5 mol% isopropyl alcohol/CO_2 [292].

A recent paper reported the separation of mixtures of nickel and vanadyl porphyrins, and of two samples from nature (an extract of ordinary green grass and Maya asphaltene) with 20% methanol-modified CO_2, 4.6-mm i.d. packed

columns, and UV detection [293]. The C_{18} stationary phase proved to be more selective for the separation of porphyritic derivatives than were silica and phenyl stationary phases. Nickel and vanadyl porphyrin chelates were separated successfully by both sub- (50°C) and supercritical (100°C) fluid chromatography at constant pressure (ca. 270 atm). The results of this study demonstrated the feasibility of applying SFC to the examination of metalloporphyrin mixtures after careful optimization of conditions.

In early studies dealing with the separation of metal acetylacetonates and thenoyltrifluoroacetonates by SFC, dichlorodifluoromethane was used as the mobile phase [294, 295]. The successful elution of about 65 β-diketonate chelates has been published in the literature [66, 294–297]. Resolution by SFC on packed columns is usually not very good and peaks are broak, but a mixture of three Cr(III) β-diketonates has nevertheless been separated with excellent resolution [296]. The tailing of peaks so often seen in SFC chromatograms of these compounds could be due to adsorption on the stationary phase or to decomposition during the SFC run.

6.13 Enantiomers

The requirement for chiral purity in the pharmaceutical industry is becoming stricter, which places greater demands on the analysis of reagents and products of asymmetric synthesis, fluids containing enantiomeric drugs for pharmacokinetic studies, and environmental samples for which the determination of the fate of specific enantiomers in synthesis, biological systems, and the environment is important. As the requirements for chiral purity in the pharmaceutical industry become stricter, the means of separation of enantiomers becomes more important.

Analytical SFC plays an important role in the separation of enantiomers for several reasons: (1) SFC requires relatively low temperatures compared to GC, (2) derivatization is not always necessary, (3) separations by SFC are faster and have higher resolution than can be achieved in LC, (4) open tubular column SFC is more compatible with flow sensitive detectors like the mass spectrometer than conventional LC, and (5) the cavity effects of inclusion-type chiral separation phases (CSPs) are more selective due to the properties of supercritical fluids compared to mobile phases for LC.

The early commercialization of the Pirkle-type phases for LC [298] has made them quite popular among SFC users. The first SFC chromatogram of enantiomers was obtained by using a packed column with a Pirkle-type CSP [299]. Mourier and co-workers [300] later used this phase to study retention and selectivity of methyl-1-(4-methylnaphthyl)phenyl phosphine oxide in supercritical CO_2. Macaudiére and co-workers [301] used a Pirkle-type phase to resolve amide enantiomers for the comparison of subcritical fluid chromatography (SubFC) and LC. Röder and co-workers [302] were the first to use a Pirkle-type phase in open tubular column SFC. Derivatized amino acid enantiomers were found to have lower enantioselectivities in SFC than in LC under the experimental conditions reported.

Hara and co-workers [303] developed and used an *N*-formylvaline CSP bonded to silica gel to separate α-amino acid derivatives, and a diamide CSP to separate D- and L-amino acid derivatives [304]. Later, Dobashi and co-workers [305] developed valine–diamide CSPs for the separation of racemic *N*-4-nitrobenzoylamino acid isopropyl esters in SFC. Gasparrini and co-workers [306] developed a 3,5-dinitrobenzoyl derivative of *R,R*(−)-1,2-diamino-cyclohexane covalently bonded to silica gel and resolved enantiomeric sulfoxides and racemic alprenolol oxazolidin-2-one under subcritical fluid conditions.

Type-II CSPs are phases that include polymers having several chiral centers, such as those described by Ichida and co-workers [307] and Shibata and co-workers [308]. A chiral separation of α-methylene lactam on a cellulose tribenzoate CSP (Chiralcel OB) was achieved by Caude and Macaudière [309] using subcritical CO_2 with an isopropyl alcohol modifier [92:8 (w/w)] at 25°C. Due to low efficiency, this degree of resolution could not be obtained in LC.

Petersson and co-workers [310] reported the development of a cyclohexyldiamide polysiloxane copolymer for the resolution of several underivatized cyclic, linear, aromatic, and aromatic diol enantiomers, which fits into the Type-II category. The copolymer is made up of copolymerized chiral and achiral monomers resulting in positions in the polymer backbone with a chiral twist. Functional groups on the copolymer interact most readily with the enantiomer

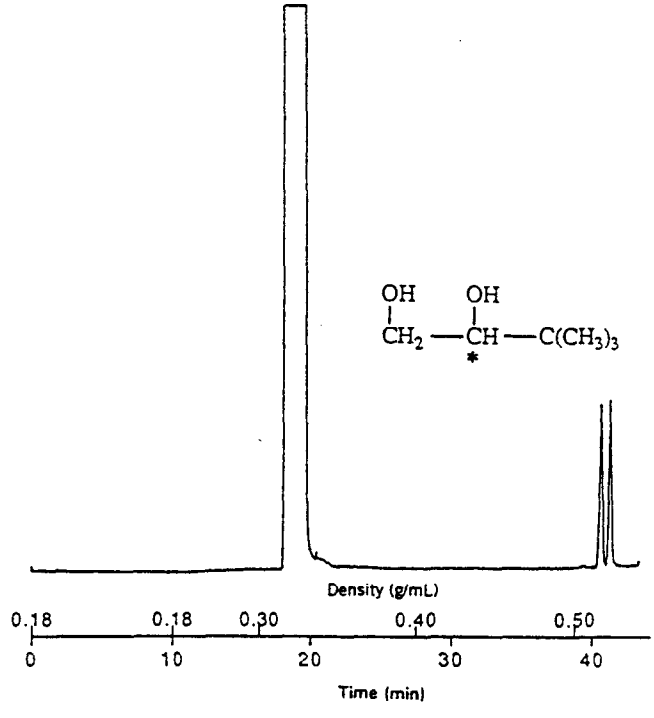

Figure 1.35 An SFC chromatogram of racemic 3,3-dimethyl-1,2-butanediol. Conditions: 20-m × 50-μm i.d. open tubular column with a cyclohexyldiamide polysiloxane copolymer stationary phase (d_f = 0.20 μm); CO_2 at 50°C; multilinear density program from 0.18 to 0.57 g/mL over 46 min; FID. Reprinted with permission from P. Petersson, D. Johnson, M. Eguchi, K. E. Markides, B. Rossiter, J. S. Bradshaw, and M. L. Lee, *J. Microcol.* Sep. **4**, 155 (1992).

that fits best into these "grooves." Figure 1.35 shows a successful separation of chiral diols using this CSP.

The Type-III phases include those CSPs that involve the formation of inclusion complexes. The phenylmethacrylate CSPs reported by Okamoto and Hatada [311] and by Blaschke [312], the cyclodextrin CSPs described by Armstrong and DeMond [313], and the microcrystalline cellulose triacetate CSP reported by Hesse and Hagel [314] fit into this category.

Macaudiére and co-workers [315] were the first to report the use of β-cyclodextrin-bonded stationary phases for the resolution of racemic amides and phosphine oxides in packed column subcritical fluid chromatography. Figure 1.36 shows a comparison of the enantiomeric separations of 2-naphthyl and *o*-

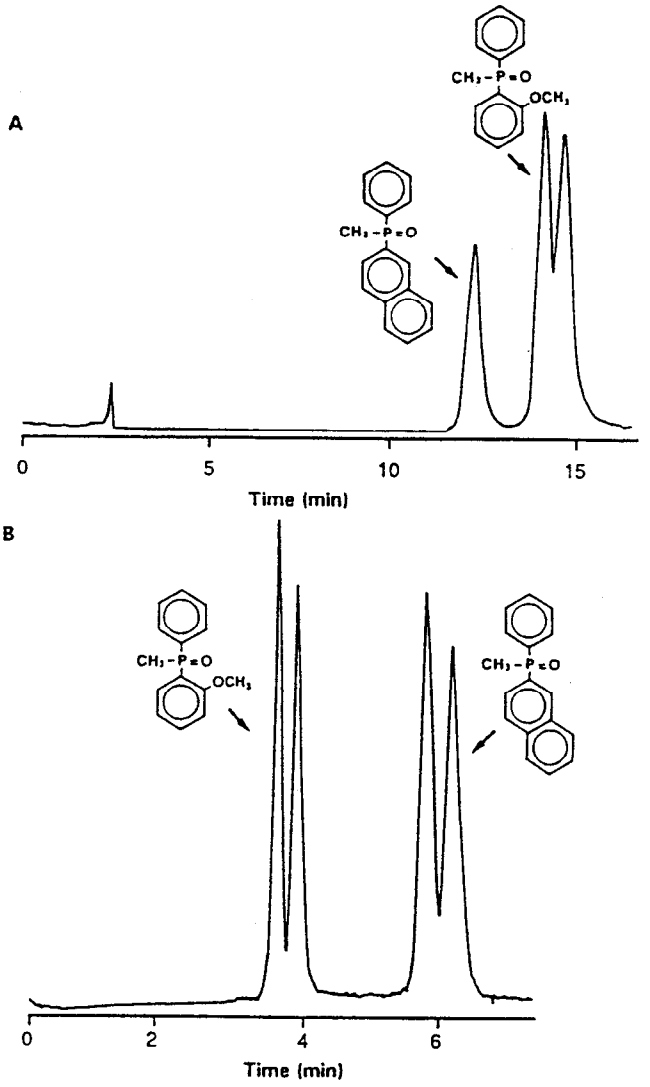

Figure 1.36 The SubFC and LC chromatograms comparing the resolution of the racemic 2-naphthyl and *o*-anilyl phosphine oxides. Conditions: 25-cm × 4.6-mm i.d. packed column with a β-cyclodextrin-bonded (Cyclobond I) phase on 5-μm particles: (*A*) ethanol/hexane LC mobile phase at 1 mL/min; (*B*) 8:92 (w/w) methanol/CO_2 SubFC mobile phase at 4.5 mL/min, 25°C, 150 atm; UV (234 nm). Reprinted with permission from P. Macaudiére, M. Caude, R. Rosset, and A. Tambuté, *J. Chromatogr.*, **405**, 135 (1987).

anisyl phosphine oxides on a β-cyclodextrin bonded CSP (β-CD) in LC and subFC. Macaudiére and co-workers [315] found that the free volume of the β-CD cavity is modified by the type of mobile phase. Since the CO_2 molecule (the subFC mobile phase) is smaller than the hexane molecule (the LC mobile phase), it is more easily displaced from the β-CD cavity. The stereoselectivity (α) for LC was found to be consistently lower than in subFC. Also, because the 2-naphthylphosphine oxide could form inclusion complexes in subFC, it was resolved and eluted after the *o*-anisylphosphine oxide. However, the 2-naphthylphosphine oxide eluted more rapidly than the *o*-anisylphosphine oxide and was unresolved using the hexane LC mobile phase. Attempts to resolve these compounds using reversed-phase (water/methanol, 75:25 or 50:50) mixtures in LC also failed. They reported that in normal phase LC, solvents such as *n*-hexane and chloroform are not readily displaced from the cyclodextrin cavity. However, the small size of the CO_2 molecule allowed it to be more readily displaced by the eluting solutes. Later, these authors [316] showed that retention using β-cyclodextrin CSPs was dependent on the nature and content of the modifier, the pH of the mobile phase, and the buffer concentration.

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